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ORIGINAL ARTICLE

Stable Carbon Isotope Composition of *c*9,*t*11-Conjugated Linoleic Acid in Cow's Milk as Related to Dietary Fatty Acids

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Abstract This study explores the potential use of stable carbon isotope ratios ($\delta^{13}C$) of single fatty acids (FA) as tracers for the transformation of FA from diet to milk, with focus on the metabolic origin of *c*9,*t*11-18:2. For this purpose, dairy cows were fed diets based exclusively on C_3 and C_4 plants. The FA in milk and feed were fractionated by silver-ion thin-layer chromatography and analyzed for their $\delta^{13}C$ values. Mean $\delta^{13}C$ values of FA from C_3 milk were lower compared to those from C_4 milk (−30.1‰ vs. −24.9‰, respectively). In both groups the most negative $\delta^{13}C$ values of all FA analyzed were measured for *c*9,*t*11-18:2 (C_3 milk = −37.0 ± 2.7‰; C_4 milk −31.4 ± 1.4‰). Compared to the dietary precursors 18:2n-6 and 18:3n-3, no significant ^{13}C-depletion was measured in *t*11-18:1. This suggests that the $\delta^{13}C$-change in *c*9,*t*11-18:2 did not originate from the microbial biohydrogenation in the rumen, but most probably from endogenous desaturation of *t*11-18:1. It appears that the natural $\delta^{13}C$ differences in some dietary FA are at least partly preserved in milk FA. Therefore, carbon isotope analyses of individual FA could be useful for studying metabolic transformation processes in ruminants.

Keywords $\delta^{13}C$ values · Vaccenic acid · Milk · α-linolenic acid · C_3 plants · C_4 plants

Abbreviations

Ag^+-TLC	Silver-ion thin-layer chromatography
CLA	Conjugated linoleic acid
CSIA	Compound-specific isotope analysis
$\delta^{13}C$ values	Stable carbon isotope composition
FA	Fatty acid(s)
FAME	Fatty acid methyl ester(s)
GC/C/IRMS	Gas chromatography-combustion-stable isotope ratio mass spectrometry
MUFA	Monounsaturated fatty acid(s)
PUFA	Polyunsaturated fatty acid(s)
VPDB	Vienna Pee Dee Belemnite standard

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Introduction

Diet formulation for dairy cows is the most efficient tool to enhance milk quality strategically in terms of lipid composition. However, a substantial amount of FA not present in the diet is formed by microbial activity in the rumen during passage of the lipids through the digestive tract. Maintaining the delicate balance of intact transfer of desired FA to the milk and its enrichment with favorable primary or secondary products of ruminal biohydrogenation is an important issue of research in ruminant nutrition [1]. For humans ruminant-derived foods are the major source of conjugated linoleic acids (CLA) [2, 3], a group of

positional and geometric isomers of octadecadienoic acid with conjugated double bonds [4]. *Cis*-9, *trans*-11 octadecadienoic acid (*c*9,*t*11-18:2; rumenic acid) is the main isomer in ruminants and represents up to 90% of total CLA in cow's milk [5] but its concentration depends on the diet. Especially this isomer is supposed to have anti-carcinogenic [6, 7], anti-atherogenic [8, 9], and anti-diabetic effects [10]. It therefore gained attention both in human [11] and animal nutrition research [12, 13].

The concentration of *c*9,*t*11-18:2 depends on the dietary proportions of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) [14] as it occurs as an intermediate in the rumen during microbial biohydrogenation of these polyunsaturated FA (PUFA). During this process 18:2n-6 originating from the diet is first isomerized at the *cis*-12 double bond, producing *c*9,*t*11-18:2, which is then converted to vaccenic acid (*t*11-18:1) [4, 15]. Similarly, 18:3n-3 first undergoes an isomerization step to *c*9,*t*11,*c*15-18:3, which is followed by a hydrogenation of the *cis*-double bonds to *t*11-18:1 [4]. This intermediate of 18:3n-3 and 18:2n-6 biohydrogenation can endogenously be desaturated to *c*9,*t*11-18:2 by Δ^9-desaturase in the tissue. It has been estimated that >60% of *c*9,*t*11-18:2 in milk fat originate from desaturation and the remaining <40% directly from microbial isomerization [4, 16]. Thus, the metabolic availability of *c*9,*t*11-18:2 in ruminants depends on (i) the extent of ruminal production of *c*9,*t*11-18:2 and *t*11-18:1, determined by nutritional factors and ruminal biohydrogenation processes [12, 14, 17], and (ii) the extent of endogenous desaturation of *t*11-18:1 to *c*9,*t*11-18:2 [16].

To be able to influence concentrations of *c*9,*t*11-18:2 strategically in ruminant-source foods it is necessary to know the proportions of this FA which originate from 18:2n-6 or 18:3n-3. This can be accomplished by using specifically labeled FA as metabolic tracers. DeNiro and Epstein [18] showed that the isotopic composition (δ^{13}C values in ‰ vs. Vienna Pee Dee Belemnite standard, VPDB) of the body of animals is determined by their diet, with a ^{13}C enrichment (trophic shift) of about 1‰. Therefore, stable isotope ratios of FA have been used before in animals when dealing with ecological [19–21], traceability [21–23], and metabolic research questions [24–26]. Using specifically labeled FA for oral administration in large animals is a very expensive approach, and artificial diet components may have to be used. Alternatively, the natural difference between feeds from C_3 and C_4 plants, differing in their stable carbon isotope composition might be utilized. This approach has been successfully applied in transfer studies in ruminants [27]. Molkentin et al. [27] reported ^{13}C-depleted organic milk as compared to conventional milk as a consequence of a wide exclusion of maize as a forage plant in this production system. The metabolism of C_4 plants like maize using the Hatch-Slack cycle discriminates less against ^{13}C during photosynthetic fixation of atmospheric CO_2 (δ^{13}C of −16 to −9‰), while C_3 plants such as temperate climate grasses and cereals follow the Calvin-Benson cycle (δ^{13}C of −34 to −22‰) [28]. Since various feeds from C_3 and C_4 plants are available, a less expensive approach to evaluate the metabolic origin of FA in ruminant products could be developed on this basis. Indeed, differences in δ^{13}C values have been found between individual FA of various plant species [29–36]. For methyl branched FA, de novo synthesized by microorganisms, clear differences in the δ^{13}C values from those of straight-chain FA have been shown [26]. However, the rumen microbial influence on the isotopic composition of FA, which are only hydrogenated and isomerized in the rumen, is not yet documented.

In the present study we explore the possible use of naturally occurring differences in the C isotope composition of individual long-chain FA between C_3 and C_4 plant species to trace the digestive and metabolic fate of these FA in ruminants. This would offer an approach to quantify the relative contribution of diet components to the pools of long-chain FA used for tissue (meat) and milk synthesis. In particular, this study aims at evaluating changes in the δ^{13}C values between dietary 18:2n-6 or 18:3n-3 and *c*9,*t*11-18:2 in milk fat, which may originate from endogenous desaturation in the tissues and/or from microbial isomerization in the rumen. This should elucidate whether such an approach can give useful information for the description of *c*9,*t*11-18:2 synthesis pathways. The separation of FA groups by silver-ion thin-layer chromatography (Ag^+-TLC) prior to compound-specific isotope analysis (CSIA) of individual FA with gas chromatography-combustion-stable isotope ratio mass spectrometry (GC/C/IRMS) could be a useful approach to trace transformations of dietary FA in ruminants digestion (biohydrogenation of PUFA) and endogenous processes (chain-elongation of dietary PUFA). Here we compare the stable C isotope composition of individual FA from two experimental diets based on C_3 and C_4 plants with those extracted from the milk of cows consuming these diets.

Materials and Methods

Animals and Diets

Two isoenergetic and isonitrogenous diets were designed and fed to dairy cows that were in mid to end of lactation and having a limited milk yield of on average 15 kg/day. In this stage of lactation cows were assumed to be in a steady or anabolic phase and to be not mobilizing body lipids. Six cows received a diet composed of feeds obtained only from C_3 plants. These included [g/kg dry matter (DM)] barley

straw (459 g/kg dry matter, DM), barley grain (266 g/kg DM), soybean meal (238 g/kg DM) and sugar beet molasses (21 g/kg DM). Another five animals received a diet composed exclusively of C_4 plant feeds, namely maize straw (444 g/kg DM), maize pellets (368 g/kg DM), maize gluten (151 g/kg DM) and sugar cane molasses (22 g/kg DM). Both diets were supplemented by minerals, vitamins and some urea. The C_3 and C_4 diets contained 18.6 and 36.0 g/kg DM total fat and were balanced in net energy for lactation (5.46 MJ/kg DM) and protein (217 and 211 g/kg DM, respectively). At the start of the experiment, the diet was changed stepwise within 6 days from hay ad libitum and 3 kg/day of barley grain to the experimental diets. Data and sample collection did not start before a further 14 days had passed, thus minimizing carry-over effects in C-isotope composition from the previous diet. Then feed intake and milk yield were measured daily, and milk samples were collected every morning and evening for another 8 days. These samples were pooled per cow. Feed samples were collected two times. The feed samples were homogenized and pulverized and stored in 150 mL PET flasks at room temperature until analysis. Milk fat was obtained by centrifugation of milk samples and stored in the dark at −20 °C. For analysis, milk fat samples were thawed at room temperature. The governmental veterinary authority for animal welfare approved the animal experiment. Further information about diets and other experimental details are given elsewhere [14, 24].

Lipid Extraction from Feed Samples

Aliquots of 2–3 g of each compound of the powdered feed samples (except molasses), were weighed in metallic extraction thimbles (Dionex Corporation, Sunnyvale, CA, USA). Lipids were extracted with hexane/isopropanol (3:2, v/v) over night by accelerated solvent extraction (ASE 200, Dionex Corporation, Sunnyvale, CA, USA) [37]. The solvent was then evaporated under a N_2 stream and residues were dissolved in dichloromethane. Evaporation under N_2-atmosphere was repeated and the lipid extract was saponified as described below.

Saponification of Lipid Extracts and Milk Fat, and FA Derivatization

The lipids in the samples were saponified with methanolic sodium hydroxide. For FA conversion, methanolic boron trifluoride was used according to IUPAC method 2.301 [38]. After boiling, approximately 100 mg of pure fat with 2 mL NaOH (0.5 M) for 3 min, 3 mL methanolic boron trifluoride (1.3 M) was added and the mixture was heated again for 4 min. The reaction was stopped by adding 7 mL NaCl (0.34 M) and 2 mL hexane. Subsequently, tubes were shaken for 30 s and centrifuged at 1,100×*g* for 1 min. A 1 mL aliquot of the upper layer, containing the FA methyl esters (FAME), was cleaned (e.g., removal of dyes) in a solid phase extraction column filled with silica gel (Isolute, Biotage, Cardiff, UK). The FAME were eluted with 6 mL dichloromethane and stored in three 2-mL vials with a final total FA concentration of approximately 20 mg/mL.

Silver-Ion Thin-Layer Chromatography

The Ag^+-TLC was used to separate saturated from mono- and from polyunsaturated FAME. This step was necessary to improve C18 FA (e.g., *cis*-18:1 and *trans*-18:1) separation in compound-specific C-isotope analysis. The Ag^+-TLC was applied for all samples as described by Richter et al. [39]. The TLC silica gel glass plates (60F-254 glass plates, 20 × 20 cm, MERCK, Darmstadt Germany) were first conditioned in TLC tanks filled with chloroform/methanol (1:1, v/v). After impregnation with 10% $AgNO_3$ in acetonitrile, plates were dried at 110 °C and 500 µg FAME were applied as a narrow linear band in the lower part of the plate. The mobile phase contained toluene/hexane (1:1, v/v). After the mobile phase reached the area between the borders of silver-ion and stationary phase, plates were dried at room temperature for 1 h. Four FAME bands, representing PUFA, monounsaturated FA (MUFA), *trans* FA (TFA) and saturated FA (SFA) were visualized by spraying with 2,7-dichlorfluorescein in ethanol. Each band was detached by scraping and washed with 4 mL chloroform/methanol (9:1, v/v) into filtration tubes filled with sodium sulfate and fiberglass. Eluents were dried under nitrogen and residues were dissolved in 200 µL hexane and stored at +4 °C for further analyses.

FA Analysis by GC/MS and GC/FID

In advance of CSIA, GC/MS and GC/FID analyses were performed in order to identify and quantify the individual FA. The analysis of FA in feed and milk fat was performed in duplicate on a gas chromatograph (GC; Agilent 6890 Series GC-Systems, Wilmington, DE, USA) equipped with a 30 m × 320 µm × 0.25 µm Supelcowax-10 column (Sigma Aldrich, Bellefonte, PA, USA) and a flame ionization detector. Helium was used as carrier gas with a constant flow of 1.1 mL/min. Samples were injected at a temperature of 200 °C and a split of 10:1. The oven temperature program was 2 min at 150 °C, +5 °C/min up to 160 °C for 5 min, +10 °C/min up to 190 °C for 5 min and +3 °C/min up to 250 °C for 5 min and the total time was 42 min. Individual FAME were identified by comparison of retention times with those of a standard FAME mixture (Supelco 37 component FAME Mix, Inc., Bellefonte, PA,

USA), after characterization of the single FA by gas chromatograph–mass spectrometry (Thermo Fisher, Argenteuil, France) equipped with a Supelcowax-10 column (see above). Chromatograms were evaluated by using the HP ChemStation software (Hewlett Packard, Palo Alto, CA, USA).

Carbon Isotope Analyses of FA Using a Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometer

The $\delta^{13}C$ values of individual FA were obtained by using an Agilent 6890 GC coupled to a Thermo Fisher (Bremen, Germany) Delta V isotope ratio mass spectrometer (IRMS) by a combustion (C) interface III (GC/C/IRMS) under a constant helium flow of 1.1 mL/min. The combustion interface consists of two ceramic furnaces: an oxidation reactor with CuO/NiO/Pt wires at 940 °C and a reduction reactor with Cu wires at 600 °C. Water was removed from the effluent gas by passing it through a Nafion tube (Perma Pure, Toms River, NJ, USA) with an annular back-flow of helium. The GC was operated with the same type of column (Supelco-Wax 10 column) and temperature program used for GC/FID analyses. The background subtraction and $\delta^{13}C$ values calculation were performed using the Thermo Fisher ISODAT 2.5 software. The standard deviations for repeatability ranged between 0.03 and 1.0‰ for the main FAME. The accuracy of the GC/C/IRMS analyses was checked every 10 analyses by injection of a 20:0 methyl ester isotope standard prepared by A. Schimmelman from the Biogeochemical Laboratories at Indiana University, USA. The isotopic shift due to the carbon introduced in the FA methylation was corrected by a mass balance equation [33]: $\delta^{13}C_{FAME} = f_{FA}\delta^{13}C_{FA} + f_{MeOH}\ \delta^{13}C_{MeOH}$ where $\delta^{13}C_{FAME}$, $\delta^{13}C_{FA}$ and $\delta^{13}C_{MeOH}$ are the C-isotope compositions of the FAME, the FA, and the methanol used for methylation of the FA, respectively, and f_{FA} and f_{MeOH} are the C-fractions in the FAME due to the alkanoic chain and methanol, respectively. Since *trans* 18:1 isomers elute close to each other, the $\delta^{13}C$ value reported as *t*11-18:1* include isomers *t*9, *t*10, *t*11 with *t*11 being by far the most abundant (e.g., in the milk from C_3 fed cows *t*11-18:1 ~71.3%; in the milk from C_4 fed cows *t*11-18:1 ~81.2%) [14].

Statistical Evaluation

Isotopic values for total diets were calculated based on proportionate intakes of individual FA from the individual feeds [14]. To compare the stable C isotope composition of the diets at two different sample collection dates, a *t*-test was carried out on the individual FA of the diet components, but no significant differences (data not shown) between the sampling dates were found. The isotopic values of the individual FA in the three C_3 feeds and the three C_4 feeds, as well as of the six and five milk samples were subjected to analysis of variance using the general linear model (GLM procedure; SAS software, version 9.1, SAS Institute Inc., Cary NC, USA), considering as effects individual FA and diet type and the interaction. Multiple comparisons among FA means in the feeds were performed using Tukey's method. Mean values of individual milk FA within one diet group were compared by the least significant difference test ($p < 0.05$).

Results

Carbon Isotopic Composition of the FA in the C_3 and C_4 Feeds

The carbon isotope composition measured in the FA of individual feeds and calculated for the total C_3 and C_4 diets are given in Table 1. All the FA $\delta^{13}C$ values in C_3 feeds (mean value: −32.3‰) were more negative ($p < 0.001$) than those in C_4 feeds (mean value: −23.4‰). In both diet types, the lower $\delta^{13}C$ values were measured in 18:0 and FA with <18 C-atoms compared to the unsaturated C18 fatty acids (Table 1). Within the groups of isotopically light FA, there was a further differentiation for 18:0, which had a more negative $\delta^{13}C$ than 16:0 and 16:1. No significant interaction between feed type and FA occurred for $\delta^{13}C$ values.

Carbon Isotopic Composition of FA in Cow's Milk

The mean $\delta^{13}C$ values of the main FA of milk samples from animals fed with C_3 or C_4 diets were lower compared to those of C_4 milk (−30.1 ± 4.4‰ and −24.9 ± 3.8‰, respectively; Table 2). Significant differences in $\delta^{13}C$ of most FA (except 14:1, 16:1, 17:1 and 18:3n-3) were observed between milk samples from C_3 and C_4 diets. Within a diet group, differences ($p < 0.001$) in $\delta^{13}C$ values between the main FA were found. In both groups the lowest $\delta^{13}C$ values were measured for *c*9,*t*11-18:2 (C_3 milk = −37.0 ± 2.7‰; C_4 milk = −31.4 ± 2.6‰; Table 2). A trend was identified in the $\delta^{13}C$ values of C18 FA in C_3 milk. They were enriched in ^{13}C with increasing degree of desaturation (18:0 < 18:1n-9 and *t*11-18:1 < 18:2n-6 < 18:3n-3), with the exception of *c*9,*t*11-18:2 (Table 1). In C_4 milk, such enrichment was only observed for *t*11-18:1 and 18:2n-6. The $\delta^{13}C$ values of 18:1n-9 and *t*11-18:1 were not significantly different in both groups (C_3 milk: −31.8 ± 1.4 and −30.6 ± 2.6‰, C_4 milk: −26.5 ± 2.1 and −23.4 ± 1.4‰). Different results between groups were observed for 18:3n-3, which had lower $\delta^{13}C$ values than *t*11-18:1 in C_4 milk but higher $\delta^{13}C$

Table 1 Means for stable carbon isotope composition in individual fatty acids from the diet (δ^{13}C, ‰ VPDB)

	C_3 diet				C_4 diet				Average δ^{13}C values		
	Total diet^{+}	Barley straw	Barley grain	Soybean meal	Total diet^{+}	Maize straw	Maize pellets	Maize gluten	C_3 feeds	C_4 feeds	*p*-value C_3 versus C_4
16:0	−34.0	−35.2	−34.3	−32.1	−23.9	−26.3	−23.6	−22.8	−33.9^{c}	−24.2^{b}	<0.001
16:1	−33.2	−33.1	−33.9	−32.0	−24.7	−24.5	−26.0	−23.6	−33.0^{bc}	−24.7^{b}	<0.001
18:0	−36.8	−40.3	−37.3	−34.3	−26.3	−28.2	−25.8	−25.3	−37.3^{d}	−26.4^{b}	<0.001
18:1n-9	−30.0	−31.5	−30.9	−28.2	−21.0	−20.4	−21.2	−20.6	−30.2^{ab}	−20.7^{a}	<0.001
18:2n-6	−29.4	−30.2	−30.2	−27.8	−19.9	−18.6	−19.8	−20.5	−29.4^{a}	−19.6^{a}	<0.001
18:3n-3	−30.5	−31.4	−31.6	−27.8	−17.2	−22.7	−16.8	−15.0	−30.3^{ab}	−18.1^{a}	<0.001
p-value fatty acids									<0.001	<0.001	

$^{+}$ Total C_3 and C_4 diets were determined by mass balance calculations from the proportion of fatty acid intake from the three dietary components

^{a,b,c,d} Fatty acid means within a column with different superscript letters are significantly different ($p < 0.05$)

Table 2 Means and ranges for stable carbon isotope composition in individual milk fatty acids (δ^{13}C, ‰ VPDB)

Milk origin	From cows fed the C_3 diet ($n = 6$)	From cows fed the C_4 diet ($n = 5$)	*p*-value diet
12:0	−28.8 (−31.5 to −25.9)	−22.6 (−24.9 to −20.7)	<0.001
14:0	−32.3 (−32.8 to −31.5)	−23.2 (−24.6 to −21.5)	<0.001
14:1	−27.0 (−29.0 to −22.4)	−22.9 (−27.1 to −19.9)	0.044
15:0	−35.0 (−37.5 to −34.2)	−27.0 (−29.0 to −25.3)	<0.001
16:0	−29.6 (−31.0 to −28.4)	−22.3 (−23.8 to −21.0)	<0.001
16:1	−26.8 (−29.8 to −22.6)	−24.4 (−29.6 to −20.2)	0.274
17:1	−26.3 (−29.2 to −21.2)	−24.4 (−29.6 to −20.2)	0.352
18:0	−34.6 (−36.5 to −32.6)	−26.6 (−29.3 to −23.5)	<0.001
18:1n-9	−31.8 (−34.0 to −29.8)	−26.5 (−29.1 to −23.6)	<0.001
*t*11-18:1*	−30.6 (−33.2 to −25.6)	−23.4 (−25.2 to −21.2)	<0.001
18:2n-6	−29.3 (−36.7 to −26.8)	−22.4 (−25.9 to −20.2)	<0.005
18:3n-3	−26.5 (−29.0 to −24.5)	−26.0 (−33.8 to −19.4)	0.818
*c*9,*t*11-18:2	−37.0 (−40.4 to −32.5)	−31.4 (−35.3 to −29.1)	0.007
Least significant difference	2.73	3.41	
p-value fatty acids	<0.001	<0.001	

* Proportions of the *trans* 18:1 isomers in %: milk from the C_3 diet: *t*9 = 14.9%, *t*10 = 13.8%, *t*11 = 71.3%; milk from the C_4 diet: *t*9 = 10.2%, *t*10 = 8.5%, *t*11 = 81.2% [14]

values in C_3 milk. There was an interaction between milk samples from different diet types and individual FA in δ^{13}C ($p = 0.002$).

Relationships of δ^{13}C Values of Individual FA Between Feed and Milk

The 16:0 was ^{13}C enriched in milk fat by 4.4‰ when fed the C_3 diet and by 1.6‰ with the C_4 diet relative to the plant 16:0 (Tables 1, 2; Fig. 1). Mean δ^{13}C values of milk 16:1 were similar to those of the C_4 diet, and 6‰ higher for the C_3 diet. The δ^{13}C values of 18:3n-3 and 18:0 in milk from C_3 fed cows were higher (−26.5 ± 1.5 and −34.6 ± 1.4‰, respectively) than for the FA of the calculated total C_3 diet (−30.5 and −36.8‰ respectively; Fig. 1). The δ^{13}C value of 18:2n-6 from C_3 diet and milk samples were similar (−29.3 ± 3.7‰ and –29.4‰, respectively). For *c*9,*t*11-18:2 the value was much lower (−37.0 ± 2.7‰) than for its precursors 18:2n-6 (−29.4‰) and 18:3n-3 (−30.5‰) in the C_3 diet.

The δ^{13}C values for 18:3n-3 in the C_4 milk samples were up to 9‰ lower than those calculated by mass balance from the total C_4 diet (milk = −26.0 ± 5.5‰, diet = −17.2‰; Fig. 1). The isotopic composition of 18:0 in the milk (−26.6 ± 3.7‰) was similar to the calculated total C_4 diet (−26.3‰, Fig. 1), and for 18:2n-6 the δ^{13}C values of the milk samples (−22.4 ± 2.2‰) were always lower. The highest intake of 18:3n-3 and 18:2n-6 was from maize pellets, due to the comparatively higher total fat content. The isotopic value of *c*9,*t*11-18:2 (−31.4 ± 2.6‰) was much lower than for its precursors 18:2n-6 (−19.9‰) and 18:3n-3 (−17.2‰) in the C_4 diet.

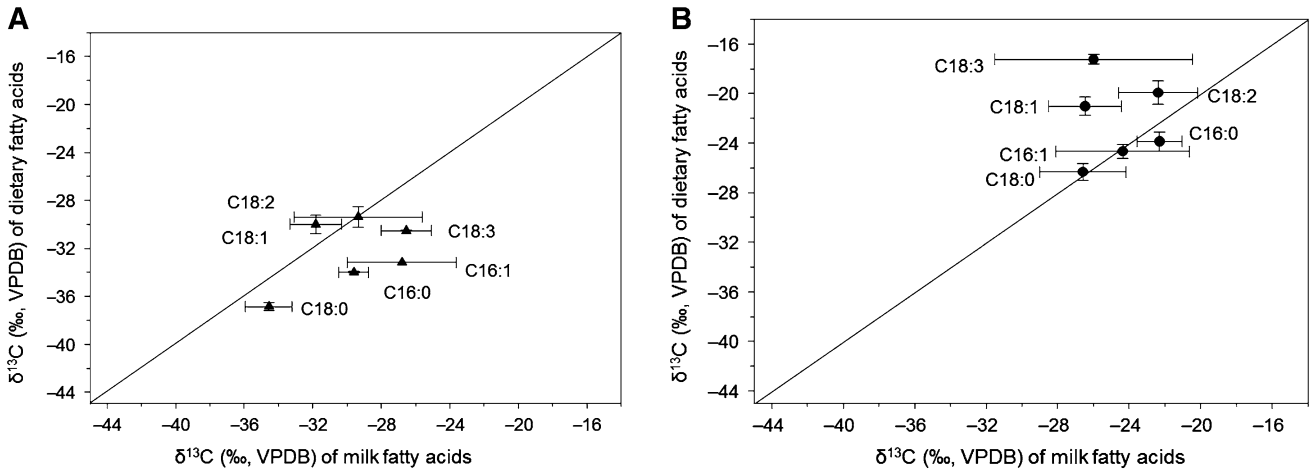

Fig. 1 Mean and standard deviation of $\delta^{13}C$ values (‰, VPDB) of individual fatty acids in the milk samples as opposed to those of the C_3 (**a**) and the C_4 diet (**b**). The isotopic composition of the total C_3 and C_4 diets were determined by mass balance calculations

Discussion

$\delta^{13}C$ Values of Individual FA in Feeds and Diets Derived from C_3 and C_4 Plants

Significant isotopic differences between (1) a specific FA in different plant species (e.g., C_3 versus C_4 plants), or (2) between FA of the same plant or (3) both are required to be able to trace transformations of FA in digestion and metabolism through natural stable C isotope compositions. A C isotope discrimination between C_3 and C_4 plants is well documented for total biomass [28] and for individual FA [29–31, 33–36, 40]. The $\delta^{13}C$ values measured in the experimental feeds for individual FA were in good agreement with these published values. The isotopic difference between 16:0 and 18:0 in the C_3 and C_4 feeds was 3.4 and 2.2‰, respectively. In both C_3 and C_4 feeds the unsaturated C18 fatty acids were depleted in ^{13}C on average by about 8‰ compared to 18:0. These differences are higher than those reported for pure vegetable oils [29–36].

Differentiation was not significant between 18:3n-3 and its precursor FA 18:1n-9 and 18:2n-6 with feeds from both C_3 and C_4 plants. In plants, 18:0 is used for the production of 18:1n-9, which is synthesized by Δ^9-desaturase. The 18:1n-9 can then be desaturated to 18:2n-6 (via Δ^{12}-desaturase) at a different site of the plant cell and a further desaturation step produces 18:3n-3 (via Δ^{15}-desaturase). These biosynthetic pathways may explain the small variations found in the $\delta^{13}C$ values. However, the highest $\delta^{13}C$ values compared to the other C18 FA were observed in the 18:3n-3 extracted from maize gluten and pellets (made from maize grains), but not in the maize straw composed mainly of leaves and stem. This ^{13}C enrichment might have resulted from the preferential cleavage of ^{12}C–^{12}C bonds over ^{12}C–^{13}C bonds during oxidation [33] of 18:3n-3 triggered by the separation of gluten from corn, pellet preparation, and their storage. Such ^{13}C enrichment of 18:3n-3 compared to 18:0, 18:1n-9 and 18:2n-6 was recently reported for poppy oil [31].

$\delta^{13}C$ Values of Individual FA in Milk from Cows Fed C_3 and C_4 Diets

In the present study, the mobilization of adipose lipids for milk synthesis was considered to be negligible. Cows were in a stage of lactation and had a milk yield where energy intake can be easily covered by intake and cows typically even deposit FA in their adipose tissues. The differences measured in the isotopic composition between individual milk FA within diet groups therefore reflect initial differences in the diets, the isotopic fractionation during biosynthesis, and differences in rates of their metabolic turnover. FA in milk from ruminants originate either directly from the diet (plant FA such as 18:2n-6 or 18:3n-3), from microbial modification in the rumen (such as *trans* 18:1 isomers), from microbial synthesis in the rumen (odd- and branched-chain FA) or from de novo synthesis within the mammary gland (short-chain and medium-chain FA with 4–14 C atoms). The C16 FA can originate from both sources [41, 42], where the endogenous synthesis of 16:0 uses acetate from ruminal fermentation of carbohydrates as precursor. Even though the fat content of the C_3 total diet was lower than of the C_4 diet, in both groups of cows the 16:0 intake was similar [14]. However, the 16:0 concentration in the C_3 milk was higher [14], which suggests a higher endogenous synthesis. This is additionally supported by the ^{13}C enrichment, found in the C_3 milk fat for 16:0 and 16:1 relative to the dietary C16, which most probably reflects the isotopic composition of acetate, originating from ruminal degradation of the heavier

carbohydrates and being the endogenous precursor of C16. The bulk feed samples mainly consisted of carbohydrates and were isotopically heavier [24] than the individual fatty acids. Lipids are ^{13}C depleted relative to carbohydrates as a result of isotopic fractionation during biosynthesis [43]. The difference between the $\delta^{13}C$ values of 16:0 and 18:0 for C_3 and C_4 milk was $+5.0 \pm 1.4$ and $+4.3 \pm 1.6$‰, respectively. Apart from dietary origin, 18:0 may be produced by microbial biohydrogenation of unsaturated C18 FA being the terminal product of ruminal biohydrogenation [41]. The $\delta^{13}C$ values of 18:0 in milk from C_3- and C_4-fed cows were different by +2.2 and −0.3‰, respectively, compared with those of the dietary 18:0. This may be explained by the trophic enrichment of ^{13}C and probably microbial activity. The 18:1n-9 in milk is derived to a large proportion from desaturation of 18:0 in the mammary gland. However, 18:1n-9 in milk of cows fed the C_3 diet was enriched in ^{13}C relative to 18:0 in the milk, as it was also observed in the diets, whereas no effect was found with the C_4 diet. With the C_3 diet, the isotopic difference between 18:0 and 18:1n-9 in milk was −2.7‰ compared to −6.8‰ in the diet. These observations suggest that feeding the C_3 diet (20% 18:1n-9) [14] either caused quantitatively more 18:1n-9 to be transferred directly from the feed or that an isotopic fractionation during the endogenous synthesis of 18:1n-9 with 18:0 as a precursor took place or both. Since no such fractionation took place with the C_4 diet (15% 18:1n-9) [14], the latter seems less likely.

Dietary polyunsaturated C18 FA undergo intensive modification during ruminal digestion [12, 15]. Important intermediates and end products of these processes are *c*9,*t*11-18:2, *t*11-18:1 and 18:0 [4, 16]. Being essential, 18:2n-6 and 18:3n-3 may occur in the endogenous metabolism only as primary dietary FA. However, their $\delta^{13}C$ was not the same in feed and milk. In C_3 milk, 18:3n-3 was 4‰ enriched in ^{13}C compared to the total diet. This suggests a preferential biohydrogenation of the isotopically light 18:3n-3 molecules in the rumen followed by a transfer of the residual 18:3n-3, which is more enriched in ^{13}C to the milk. By contrast, the $\delta^{13}C$ value of 18:3n-3 in C_4-plant derived milk was much lower (about −9‰) compared to the corresponding diet. Further work is in progress to confirm these results and explain these apparently opposite isotopic trends of 18:3n-3 in milk from animals feed with C_3 or C_4 diets.

The *c*9,*t*11-18:2 was depleted in ^{13}C in milk from both diet types, compared to all other milk FA. This would suggest an isotope fractionation occurring during the isomerization step in ruminal biohydrogenation. Microbial C isotope fractionation has been observed previously for the conversion of *cis*-16:1 to *trans*-16:1 by the bacterium *Pseudomonas putida*, with the *trans*-molecule being depleted in ^{13}C by up to 2.1‰ [44]. However, in the milk of the present study, *t*11-18:1 (with minor proportions of other 18:1 *trans* isomers) did not show a comparably large deviation in $\delta^{13}C$ values from the precursors 18:2n-6 and 18:3n-3 as that found with *c*9,*t*11-18:2. Since *t*11-18:1 occurs after *c*9,*t*11-18:2 in the biohydrogenation pathway, it seems unlikely that the isotopic difference measured for *c*9,*t*11-18:2 was a consequence of the rumen microbial activity. Therefore, as *c*9,*t*11-18:2 can also be produced in the body tissue from *t*11-18:1 by Δ^9-desaturase [4, 16], the enzyme catalyzing the endogenous desaturation might discriminate against ^{13}C. This could be explained by stronger chemical bonds of the heavier isotopologues (^{12}C–^{13}C, ^{13}C–^{13}C) compared to ^{12}C–^{12}C followed by lower enzymatic rate constants [33]. Accordingly, Gilmore et al. [45] suggested that the variations in $\delta^{13}C$ values of individual FA in Arctic foxes could be attributed to isotopically distinct dietary sources and isotopic fractionation during desaturation and chain elongation. Also Fang et al. [46] assumed that a kinetic isotope effect, resulting in intermolecular isotope fractionation during desaturation and chain elongation, could be responsible for $\delta^{13}C$ differences in FA in seep organisms.

The significant interaction found between diet type and FA suggests that the differentiation in $\delta^{13}C$ values of individual FA in milk might depend on both, the diet type and the precursor FA. That implies that measurements of stable C-isotope ratios may be a useful approach to differentiate metabolic pathways of FA in ruminants if the dietary conditions are controlled.

Conclusion

By using GC/C/IRMS combined with Ag^+-TLC we were able to demonstrate natural differences between $\delta^{13}C$ values of dietary FA and milk FA. The differences in the C_3 and C_4 diets were partly preserved in the cows' milk. In particular, *c*9,*t*11-18:2 was depleted in ^{13}C compared to its dietary precursors. The study provided evidence that this was a result of the endogenous desaturation of *t*11-18:1 to *c*9,*t*11-18:2 in the body tissue rather than from rumen microbial biohydrogenation. These findings could be the basis for initiating more detailed stable isotope-based research in ruminant FA metabolism. Especially studies on various ruminant species and with different diet types are required to quantify by observing such fractionations the relative importance (species specificity, frequency, persistence) of precursors of milk FA.

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