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Effect of Diet on the Deposition of *n*-3 Fatty Acids, Conjugated Linoleic and C18:1*trans* Fatty Acid Isomers in Muscle Lipids of German Holstein Bulls

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This study examined the effects of feeding diets rich in either *n*-3 or *n*-6 polyunsaturated fatty acids (PUFA) on the fatty acid composition of *longissimus* muscle in beef bulls. Thirty-three German Holstein bulls were randomly allocated to either an indoor concentrate system or periods of pasture feeding (160 days) followed by a finishing period on a concentrate containing linseed to enhance the contents of *n*-3 PUFA and conjugated linoleic acids (CLA) in beef muscle. The relative proportion and concentration (mg/100 g fresh muscle) of *n*-3 fatty acids in the phospholipid and triglyceride fractions were significantly increased ($p \leq 0.05$) in muscle lipids of pasture-fed bulls. The pasture feeding affected the distribution of individual CLA isomers in the muscle lipids. The proportion of the most prominent isomer, CLA *cis*-9,*trans*-11, was decreased from 73.5 to 65.0% of total CLA in bulls fed on concentrate as compared to pasture. The second most abundant CLA isomers were CLA *trans*-7,*cis*-9 and CLA *trans*-11,*cis*-13 in bulls fed on concentrate and pasture, respectively. Diet had no effect on the concentration of C18:1 *trans*-11. In contrast, the concentration of the C18:1 *trans*-13/14, *trans*-15, and *trans*-16 isomers in the muscle lipids was up to two times higher in pasture-fed as compared to concentrate-fed bulls. Pasture feeding enhanced the concentration of *n*-3 fatty acids, but the diet had no effect on the concentration of CLA *cis*-9,*trans*-11.

KEYWORDS: Beef; *n*-3 fatty acids; phospholipids; trigylcerides; conjugated linoleic acid isomers; trans fatty acids

INTRODUCTION

The role of polyunsaturated fatty acids (PUFA) in human health and nutrition has refocused attention on their presence in ruminant tissues. The consumption of red meat in Europe is declining, and this is partially related to negative issues on the amount of fat and type of fat, particularly the saturated fat in the meat. The level of fat consumption and the intake of particular fatty acids, e.g., C14:0 and C16:0, and *trans* monounsaturated fatty acids in relation to certain diseases including coronary heart disease and cancer have increased the attention on ruminant meats (*1*−*4*). The fatty acid composition of ruminant tissues is complex because of fatty acid synthesis by rumen microorganisms as well as by lipolysis followed by hydrogenation of dietary PUFA to more saturated end products. Despite biohydrogenation of dietary PUFA in the rumen, some dietary unsaturated fatty acids bypass the rumen intact for absorption and deposition in intramuscular and intermuscular fat of the muscles (*5*). It is recognized that the fatty acid composition of beef muscle can be influenced by diet (*6*−*9*). Several studies have already described the potential for increasing the contents of *n*-3 PUFA in beef by feeding on fresh grass or grass silage (*6*, *8*, *10*, *11*). Increased amounts of *n*-3 PUFA in meat products from ruminants could contribute to a higher intake of *n*-3 PUFA by humans, factors recommended by nutritional guidelines for several years. It is generally suggested that the consumption of saturated and *trans* fatty acid decreases and the consumption of *n*-3 PUFA increases to achieve an *n*-6/*n*-3 ratio in the diet of approximately 5:1 or less (*12*). Besides the *n*-3 PUFA, interest in conjugated linoleic acids (CLA) has

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increased considerably over the last 10 years. CLA is a term used to describe positional and geometric isomers of linoleic acid (C18:2 *cis*-9,*cis*-12). Ruminant meats and milk and their products are the main source of CLA in the human diet. In contrast to *trans* fatty acids, CLA have been linked to a multitude of metabolic effects, including inhibition of carcinogenesis, reduced rate of fat deposition, altered immune response, and reduced serum lipids (*13*, *14*). Most of the research studies have focused on two isomers, CLA *cis*-9,*trans*-11 and CLA *trans*-10,*cis*-12 (*14*, *15*). Studies on pure single isomers showed that they have differences in biological activities as reported in recent reviews by Banni et al. (*16*) and Martin et al. (*17*). In measuring CLA, it is important to separate the individual CLA isomers because of the different effects in biological systems. The silver ion high-pressure liquid chromatography (HPLC) has proved very useful for the separation of geometrical and positional CLA isomers (*18*, *19*). CLA *cis*-9,*trans*-11 is the major CLA isomer in ruminant milk and meat products (*20*, *21*). CLA *cis*-9,*trans*-11 is formed during biohydrogenation of linoleic acid in the rumen, but the primary source is the endogenous synthesis involving Δ^9-desaturase and *trans* vaccenic acid (C18:1 *trans*-11), another intermediate in ruminal biohydrogenation (*22*, *23*).

A large study was established to investigate the effect of different breeds (German Holstein vs Simmental) and dietary regimes (concentrate vs pasture) on the meat quality and the fatty acid composition of intramuscular fat (i.m. fat) in the *longissimus* muscle of bulls. The whole study and results on aspects of meat quality and total fatty acid composition of the *longissimus* muscle have been reported by Nuernberg et al. (*24*). This paper reports the effects of the two dietary regimes on the proportion and concentration of fatty acids in muscle neutral lipid and phospholipid fractions. The study exploited the use of Ag^+ thin-layer chromatography (TLC) and Ag^+ HPLC to separate and measure individual CLA and C18:1 *trans* fatty acid isomers in the muscle lipids. Because of the complexity of the methodology, the measurements were only conducted for one breed, the German Holstein.

MATERIALS AND METHODS

Materials. Thirty-three German Holstein bulls (5−6 months old) were randomly assigned to two dietary treatments, concentrate or pasture. German Holstein bulls (17) fed on concentrate were kept in a stable and fed semi ad libitum using single fodder workstations. The concentrate ration consisted of winter barley, molasses particles, soybean extraction particles, calcium carbonate, sodium chloride, and a mixture of minerals and vitamins. The forage component of the diet consisted of maize silage (13.8 kg/day), concentrated feed (3.2 kg/day), soybean extraction particles (0.15 kg/day), hay (0.1 kg/day), and straw (0.09 kg/day). The remaining German Holstein bulls (16) were kept on pasture during two summer periods (approximately 160 days) followed by indoor periods (approximately 190 days) when animals received a semi ad libitum high-energy diet. The latter consisted of wilted silage (15 kg/day), hay (0.7 kg/day), a mixture minerals and vitamins, and a special concentrate diet containing 12% barley, 10% coarsely cracked linseed, minerals, and a mixture minerals and vitamins. The chemical composition including the fatty acid composition of the diets has been reported by Nuernberg et al. (*24*). The fatty acid compositions of the diet components grass, wilted silage, and hay contained were characterized by higher proportions of linolenic acid (C18:3 *n*-3) and lower linoleic acid (C18:2 *n*-6). Stearic acid (C18:0) contents were low in all diet components. All bulls were slaughtered at 620 kg live weight in the abattoir of the Research Institute for the Biology of Farm Animals Dummerstorf (Germany), and the carcasses were chilled (4 °C). Samples of the *longissimus* muscle were taken at the 6th rib of the left carcass side immediately and 24 h postslaughter and stored frozen at −70 °C until lipid extraction and assessment of the fatty acid composition of the muscle lipids.

Methods. *Extraction of Muscle Lipids and Separation of Lipid Classes.* The i.m. fat of 2 g of muscle was extracted with chloroform/methanol (2:1, v/v) according to Folch et al. (*25*) by homogenization (Ultra Turrax, 3 × 15 s, 12000 revolutions per minute) at room temperature. The details of muscle lipid extraction were described previously (*11*, *24*). To obtain the lipid classes, the isolated lipids were separated by TLC on precoated silica gel 60 plates (Merck, Darmstadt, Germany). The details of separation of the lipid classes were described by Lorenz et al. (*9*). Nonadecanoic acid methylester (ME) used as an internal standard was added to the lipids and the lipid classes prior to saponification and methylation.

Gas Chromatography (GC) Analysis. The fatty acid compositions of muscle lipids were determined by capillary GC using a CP SIL 88 CB column (100 m × 0.25 mm, Chrompack-Varian, United States) installed in a Perkin-Elmer gas chromatograph Autosys XL with a flame ionization detector and split injection (Perkin-Elmer Instruments, Shelton, United States). The initial oven temperature was 150 °C, held for 5 min, subsequently increased to 175 °C at a rate of 2 °C min^{-1}, held for 15 min, then to 200 °C at 7 °C min^{-1}, held for 20 min, then to 220 °C at 5°C min^{-1}, and held for 25 min. Hydrogen was used as the carrier gas at a flow rate of 1 mL min^{-1}. The split ratio was 20:1, the injector was set at 260 °C, and the detector was set at 280 °C. The amounts were calculated using the internal standard method of Turbochrom workstation software.

A reference standard "Sigma-FAME mixture" and a mixture of methyl esters of CLA were obtained from Sigma-Aldrich (Deisenhofen, Germany). Individual isomers of CLA methyl esters (CLA-ME), C18:2 *cis*-9,*trans*-11, C18:2 *trans*-9,*trans*-11, C18:2 *trans*-10,*cis*-12, C18:2 *cis*-9,*cis*-11, and methyl esters of the isomers C18:1 *trans*-6, C18:1 *trans*-9, C18:1 *trans*-11, and C18:1 *trans*-13 were purchased from Matreya (Pleasnet Gap, United States). All solvents used were HPLC grade from Lab-Scan (Dublin, Ireland). The TLC glass plates coated with 0.25 mm silica gel (20 cm × 20 cm) were obtained from Merck.

Analysis of the Trans Fatty Acids. The TLC plates were dipped into a solvent mixture of *n*-hexane/diethyl ether (9:1, v:v) for 1 h to remove all impurities from the silica gel phase. After they were air-dried, the plates were stored under drying material. The preparation of the TLC glass plates with a uniform layer of silver nitrate was performed by a dip into a 5% aqueous solution of silver nitrate (w/v) for 45 min and air-dried in the dark. The plates were stored at room temperature in the dark. Directly before chromatography, the plates were activated in the dark at 110 °C for 1.5 h. After they were cooled in the dark to ambient temperature, approximately 20 mg of fatty acid methyl esters (FAME) in *n*-heptane was applied to the plate in a narrow band. For the separation of the *cis*- and *trans*-monoenoic acids, the plates were developed with a solvent mixture of *n*-hexane/diethyl ether (9:1, v:v) for 1.5 h. After they were dried, the plates were sprayed with an ethanol solution of 2,7-dichlorofluorescein and the bands were visualized under UV light. The separated *trans* fatty acid band was scraped off, the internal standard (C19:0) was added and then two times extracted with a solvent mixture of *n*-hexane/chloroform (1:1, v:v). After that, the extracts were washed with 2 mL of a 10% solution of sodium chloride to remove trace amounts of silver nitrate and 2,7-dichlorofluorescein. Then, the extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. The extracts were stored at −20 °C until GC analysis. Analyses were performed using the same gas chromatograph and capillary column described above. The C18:1 *trans* fatty acids isomers were analyzed isothermally at 140 °C and a column head pressure of 190 kPa (split ratio 1:20). The identification of the C18:1 *trans* fatty acids isomers was made by the retention times of the standard compounds (methyl esters of the isomers C18:1 *trans*-6, C18:1 *trans*-9, C18:1 *trans*-11, and C18:1 *trans*-13). The calibration plot was used for the other isomers, also. The elution order of the C18:1 *trans* isomers was according to that used in an investigation of milk fat by Fritsche et al. (*28*) and Precht et al. (*29*).

Ag^+ HPLC. The separation of CLA isomers was performed using a HPLC system (Shimadzu, LC10A, Japan) equipped with a 50 μL injection loop, a photodiode array detector (SPD-M10Avp, Shimadzu)

operated at 233 nm, and an operating system (Shimadzu CLASS-VP version 6.12 sp4). Two ChromSpher 5 Lipids silver-impregnated columns (4.6 mm i.d. × 250 mm stainless steel; 5 μm particle size, Varian) were used in series. The mobile phase (0.1% acetonitrile, 0.5% diethyl ether in *n*-hexane) was prepared fresh daily and pumped at a flow rate of 1.0 mL/min. The injection volume depended on the total amounts of CLA and ranged between 15 and 30 μL. The determination of CLA isomers, according to the data presented in **Table 3**, was as proportions of the various isomers relative to the total area of the Ag^+ HPLC peaks. The determination was based on the measurement of integrated area under the 233 nm peaks attributed to conjugated dienes. The identification of the CLA isomers was made by the retention times of the standard compounds (C18:2 *cis*-9,*trans*-11, C18:2 *trans*-9,*trans*-11, C18:2 *trans*-10,*cis*-12, C18:2 *cis*-9,*cis*-11, see above) and the typical UV spectra of CLA isomers from the photodiode array detector (190−360 nm). The elution order of the CLA isomers in beef was reported previously by Rickert et al. (*18*) and Fritsche et al. (*21*).

Statistical Analysis. All data were analyzed by the least squares method using the GLM procedures of the StatisticalAnalysis System (SAS Systems, Release 8.2, SAS Institute Inc., Cary, NC) with fixed factor feeding. All tables contain the least squares mean (LSM) and the standard error (SE) of the LSM. All statistical tests of LSMs were performed for a significance level $p = 0.05$.

RESULTS

Fatty Acid Composition of Phospholipids (PL) and Triglycerides (TG). The i.m. fat of the *longissimus* muscle in German Holstein bulls was not affected by diet, averaging 2.63 and 2.30 g/100 g muscle on feeding concentrate and pasture. The PL content of the *longissimus* muscle varied between 0.44 and 0.68 g/100 g muscle in animals fed on pasture and concentrate, respectively. The fatty acid composition of the total lipid in *longissimus* muscle of all animals has been reported by Nuernberg et al. (*24*). The impact of diet on the fatty acid composition of the phospholipid and triglyceride fractions in *longissimus* muscle is presented in **Tables 1** and **2**.

Pasture-fed bulls had a greater proportion of total and individual *n*-3 fatty acids in the PLs as compared with those fed the concentrate diet. Linolenic acid (C18:3 *n*-3) and eicosapentaenoic acid (C20:5 *n*-3) showed a 5.0- and 3.5-fold increase, respectively, in the PL of pasture-fed bulls (**Table 1**). The proportions of C22:5 *n*-3 and C22:6 *n*-3 were increased by 1.4 and 1.9 on pasture as compared to concentrate, respectively. The *n*-3 fatty acid concentrations (mg/100 g fresh muscle) showed similar results. The total concentrations of *n*-3 fatty acids were significantly increased from 22.8 to 89.4 mg/100 g muscle in concentrate- and pasture-fed bulls, respectively (**Table 1**). The highest accumulation in the muscle of pasture-fed bulls was detected for linolenic acid. The effect of diet on *n*-3 fatty acids in the TG fraction was not as significant as compared to that noted in the PL (**Tables 1** and **2**). Pasture increased total *n*-3 fatty acids by a factor of 2.8 in the TGs (**Table 2**). The proportions of individual *n*-3 fatty acids, C18:3 *n*-3, C22:5 *n*-3, and C22:6 *n*-3, were all significantly increased.

In contrast, pasture feeding resulted in a significant decrease in the proportion of total *n*-6 fatty acids in the PLs from 32.8 to 29.1% (**Table 1**). The proportion of C20:4 *n*-6 in the PL was significantly lower, but no diet effect was detected for linoleic acid (C18:2 *n*-6). The concentration of total *n*-6 fatty acids (mg/100 g fresh muscle) was not different between the dietary treatments (**Tables 1** and **2**). In the triglyceride fraction only C18:2 *n*-6 was significantly decreased by pasture feeding (**Table 2**). The longer chain *n*-6 fatty acids were below the detection limit. Consequently, the *n*-6/*n*-3 ratio was beneficially low in pasture-fed bulls. The *n*-6/*n*-3 ratio in the PL and TG was 7.5:1 and 3.8:1 and 2.4:1 and 1.35:1 for concentrate- and pasture-fed bulls, respectively. The proportion of the total saturated fatty acids in the PLs was significantly lower in the pasture group. The proportion of C18:0 was relatively constant ranging between 12.4 and 12.5% for both diet groups. The proportion of palmitic acid was significantly decreased in the phospholipid fraction of pasture-fed bulls, as compared to concentrate-fed bulls. However, no diet effect was detected for the concentration of total saturated fatty acids in the PL and TG fraction for concentrate and pasture, respectively (**Tables 1** and **2**).

Table 1. Proportions (%) and Fatty Acid Concentrations (mg/100 g Fresh Muscle) of PL in *Longissimus* Muscle of German Holstein Bulls

	concentrate $n = 17$		pasture $n = 16$		
	LSM	SEM	LSM	SEM	*P* value
	fatty acids (%)				
C14:0	0.42	0.04	0.26	0.04	0.0123
C16:0	16.72	0.41	14.52	0.40	0.0003
C18:0	12.38	0.20	12.54	0.20	0.5855
C18:1 *cis*9	26.27	0.85	22.93	0.82	0.0067
C18:2 *n*-6	20.39	0.83	20.18	0.80	0.8566
C18:3 *n*-3	1.09	0.22	5.48	0.21	<0.0001
C20:4 *n*-6	9.39	0.31	7.02	0.30	<0.0001
C20:5 *n*-3	0.82	0.19	2.90	0.18	<0.0001
C22:5*n*-3	2.00	0.13	3.80	0.12	<0.0001
C22:6 *n*-3	0.42	0.04	0.59	0.04	0.0065
CLA *cis*-9,*trans*-11*	0.23	0.02	0.37	0.02	<0.0001
sum SFA[a]	31.33	0.46	29.90	0.44	0.0298
sum UFA[b]	68.67	0.46	70.09	0.44	0.0298
sum C18:1 *trans*[c]	0.58	0.06	0.74	0.06	0.0650
sum *n*-3 FA[d]	4.41	0.41	12.86	0.39	<0.0001
sum *n*-6 FA[e]	32.88	1.04	29.09	1.01	0.0116
n-6/*n*-3 ratio	7.52	0.24	2.42	0.23	<0.0001
	fatty acids (mg/100 g fresh muscle)				
C14:0	2.15	0.31	1.83	0.30	0.4507
C16:0	85.53	11.52	97.90	11.16	0.4440
C18:0	62.17	9.672	85.54	9.36	0.0879
C18:1 *cis*9	133.2	19.65	155.3	19.03	0.4218
C18:2 *n*-6	100.8	17.57	138.2	17.02	0.1317
C18:3 *n*-3	5.44	3.21	38.05	3.11	<0.0001
C20:4 *n*-6	47.27	7.87	47.76	7.62	0.9646
C20:5 *n*-3	4.38	2.25	20.26	2.18	<0.0001
C22:5*n*-3	10.02	2.44	26.45	2.36	<0.0001
C22:6 *n*-3	2.45	0.44	4.01	0.43	0.0151
CLA *cis*-9,*trans*-11*	1.15	0.28	2.54	0.28	0.0010
sum SFA[a]	158.4	23.03	202.9	22.30	0.1705
sum UFA[b]	345.1	56.02	478.6	54.24	0.0923
sum C18:1 *trans*[c]	3.13	0.60	4.92	0.58	0.0346
sum *n*-3 FA[d]	22.76	7.96	89.37	7.71	<0.0001
sum *n*-6 FA[e]	163.8	27.59	198.9	26.72	0.3650
n-6/*n*-3 ratio	7.27	0.22	2.42	0.21	<0.0001

[a] Sum of C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [b] Sum of C14:1 + C15:1 + C16:1 + C17:1 + C18:1 *trans* + C18:1 *cis*9 + C18:1 *cis*11 + C18:2 *trans* + C18:2 *n*-6 + C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-6 + C20:4 *n*-6 + C20:5 *n*-3 + C22:1 + C22:4 *n*-6 + C22:5 *n*-3 + C22:6 *n*-3 + *cis*9,*trans*11CLA + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-3 + C22:2 *n*-6 + C24:1. [c] Sum of the isomers C18:1*trans*6-*trans*11. [d] Sum of C20:3 *n*-3 + C22:6 *n*-3 + C22:5 *n*-3 + C20:5 *n*-3 + C18:4 *n*-3 + C18:3 *n*-3. [e] Sum of C22:2 *n*-6 + C20:2 *n*-6 + C18:3 *n*-6 + C22:4 *n*-6 + C20:3 *n*-6 + C18:2 *n*-6 + C20:4 *n*-6. *Coelution (CLA*trans*-7,*cis*-9 and CLA*trans*-8,*cis*-10).

CLA Isomers of *Longissimus* Muscle. The relative contribution of the individual CLA isomers of the *longissimus* muscle of all animals is given in **Table 3**. The predominant CLA isomer in both diets was CLA *cis*-9,*trans*-11. However, the proportion of individual CLA isomers was significantly affected by the diet. Feeding pasture as compared with concentrate significantly decreased the proportion of CLA *cis*-9,*trans*-11 in the total CLA as compared with concentrate-fed bulls from 73.5 to 65.0% (**Table 3**). The CLA *trans*-7,*cis*-9 isomer of the muscle varied

Table 2. Proportion (%) and Fatty Acid Concentration (mg/100 g Fresh Muscle) of TG in *Longissimus* Muscle of German Holstein Bulls (ND, Not Detected)

	concentrate $n = 17$		pasture $n = 16$		
	LSM	SEM	LSM	SEM	*P* value
	fatty acids (%)				
C14:0	2.98	0.10	2.81	0.11	0.2660
C16:0	28.07	0.52	25.82	0.54	0.0040
C18:0	15.57	0.56	18.69	0.57	0.0003
C18:1 *cis*9	39.80	0.72	37.81	0.74	0.0504
C18:2 *n*-6	1.73	0.10	1.39	0.11	0.0256
C18:3 *n*-3	0.24	0.04	0.93	0.04	<0.0001
C20:4 *n*-6	ND	0.00	ND	0.00	0.6998
C20:5 *n*-3	ND	0.00	ND	0.00	0.1599
C22:5 *n*-3	0.02	0.03	0.11	0.04	0.0618
C22:6 *n*-3	ND	0.00	0.15	0.07	0.1488
CLA *cis*-9,*trans*-11*	0.27	0.02	0.42	0.02	<0.0001
sum SFA[a]	48.05	0.79	49.27	0.81	0.2859
sum UFA[b]	51.94	0.79	50.72	0.81	0.2859
sum C18:1 *trans*[c]	2.24	0.17	3.11	0.17	0.0070
sum *n*-3 FA[d]	0.47	0.11	1.37	0.11	<0.0001
sum *n*-6 FA[e]	1.76	0.10	1.47	0.11	0.0619
n-6/*n*-3 ratio	3.81	0.17	1.35	0.17	<0.0001
	fatty acids (mg/100 g fresh muscle)				
C14:0	65.00	5.75	49.60	5.93	0.0672
C16:0	620.5	55.46	455.9	57.16	0.0432
C18:0	346.9	35.35	327.0	36.44	0.6957
C18:1 *cis*9	887.5	89.04	680.0	91.78	0.1100
C18:2 *n*-6	37.19	3.49	23.97	3.59	0.0106
C18:3 *n*-3	5.32	1.43	16.54	1.47	<0.0001
C20:4 *n*-6	ND	0.00	ND	0.06	
C20:5 *n*-3	ND	0.00	ND	0.00	
C22:5 *n*-3	0.40	0.62	2.03	0.64	0.0718
C22:6 *n*-3	ND	1.31	2.74	1.35	0.1521
CLA *cis*-9,*trans*-11*	5.82	0.86	7.69	0.89	0.1367
sum SFA[a]	1063	98.55	866.7	101.5	0.1693
sum UFA[b]	1156	113.9	910.9	117.5	0.1394
sum C18:1 *trans*[c]	51.57	6.47	55.34	6.67	0.6864
sum *n*-3 FA[d]	9.93	2.61	24.61	2.69	0.0002
sum *n*-6 FA[e]	38.15	3.65	25.60	3.76	0.0198
n-6/*n*-3 ratio	3.98	0.20	1.35	0.20	<0.0001

[a] Sum of C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [b] Sum of C14:1 + C15:1 + C16:1 + C17:1 + C18:1 *trans* + C18:1 *cis*9 + C18:1 *cis*11 + C18:2 *trans* + C18:2 *n*-6 + C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-6 + C20:4 *n*-6 + C20:5 *n*-3 + C22:1 + C22:4 *n*-6 + C22:5 *n*-3 + C22:6 *n*-3 + *cis*9,*trans*11CLA + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-3 + C22:2 *n*-6 + C24:1. [c] Sum of the isomers C18:1*trans*6-*trans*11. [d] Sum of C20:3 *n*-3 + C22:6 *n*-3 + C22:5 *n*-3 + C20:5 *n*-3 + C18:4 *n*-3 + C18:3 *n*-3. [e] Sum of C22:2 *n*-6 + C20:2 *n*-6 + C18:3 *n*-6 + C22:4 *n*-6 + C20:3 *n*-6 + C18:2 *n*-6 + C20:4 *n*-6. *Coelution (CLA*trans*-7,*cis*-9 and CLA*trans*-8,*cis*-10).

depending on the diet (9.4% in pasture-fed bulls and 6.0% in concentrate-fed bulls). This isomer was identified by Fritsche et al. (*21*) and Yurawecs et al. (*26*) and reported to be the second most abundant CLA isomer in milk fat and beef meat. However, this study has found that the second most abundant CLA isomer in the muscle of pasture-fed bulls was the CLA *trans*-11,*cis*-13, averaging 12.4% of total CLA. Furthermore, pasture feeding significantly increased some of the CLA *trans*,*trans* isomers (CLA *trans*-12,*trans*-14, CLA *trans*-11,*trans*-13, and CLA *trans*-9,*trans*-11). The proportion of CLA *trans*-10,*cis*-12 detected was low, averaging 3.9 and 1.9% of total CLA in concentrate- and pasture-fed animals, respectively. The CLA *cis*,*cis* isomers were below the detection limit in all muscle samples investigated. The geometrical isomer pairs of CLA *cis*,*trans*/CLA *trans*,*cis* and CLA *trans*-7,*trans*-9/CLA *trans*-8,*trans*-10 could not be separated by using a double Ag^+ HPLC column system. A partial resolution of some of the geometrical isomer pairs in beef could be achieved applying up to six silver ion HPLC columns in series (*27*).

Table 3. Distribution of Individual CLA Isomers (% ME) in *Longissimus* Muscle of German Holstein Bulls (ND, Not Detected)

	concentrate $n = 17$		pasture $n = 14$		
	LSM	SEM	LSM	SEM	*P* value
CLA*trans*-12,*trans*-14	0.94	0.17	2.97	0.18	<0.0001
CLA*trans*-11,*trans*-13	1.36	0.26	5.65	0.28	<0.0001
CLA*trans*-10,*trans*-12	0.83	0.08	0.87	0.09	0.7219
CLA*trans*-9,*trans*-11	1.58	0.16	2.15	0.17	0.0203
CLA*trans*-7,*trans*-9[a]	0.92	0.12	0.76	0.12	0.3693
CLA*trans*-11,*cis*-13	5.76	0.45	12.44	0.48	<0.0001
CLA*trans*-10,*cis*-12	3.86	0.33	1.90	0.35	0.0090
CLA*cis*-9,*trans*-11	73.51	0.96	64.98	1.03	<0.0001
CLA*trans*-8,*cis*-10	1.82	0.24	2.23	0.26	0.9769
CLA*trans*-7,*cis*-9	9.42	0.54	6.04	0.57	<0.0001
CLA*cis*-11,*cis*-13	ND	ND	ND	ND	
CLA*cis*-10,*cis*-12	ND	ND	ND	ND	
CLA*cis*-9,*cis*-11	ND	ND	ND	ND	
CLA*cis*-8,*cis*-10	ND	ND	ND	ND	

[a] Including CLA*trans*-8,*trans*-10.

Table 4. Proportion (%) and Concentrations (mg/100 g Fresh Muscle) of C18:1 *trans* Isomers in *Longissimus* Muscle of German Holstein Bulls

	concentrate $n = 16$		pasture $n = 16$		
	LSM	SEM	LSM	SEM	*P* value
i.m. fat (%)	2.67	0.24	2.30	0.25	0.3124
	C18:1 *trans* isomers (%)				
C18:1 *trans*-4	0.35	0.03	0.28	0.03	0.0919
C18:1 *trans*-5	0.32	0.02	0.24	0.02	0.0349
C18:1 *trans*-6/7/8[a]	1.77	0.06	1.03	0.06	<0.0001
C18:1 *trans*-9	4.76	0.16	3.02	0.16	<0.0001
C18:1 *trans*-10	14.05	1.24	3.76	1.24	<0.0001
C18:1 *trans*-11	41.16	1.03	49.43	1.03	<0.0001
C18:1 *trans*-12	15.16	0.83	10.18	0.83	0.0002
C18:1 *trans*-13/14[a]	12.43	0.45	17.58	0.03	<0.0001
C18:1 *trans*-15	4.82	0.22	6.86	0.22	<0.0001
C18:1 *trans*-16	5.16	0.25	7.59	0.25	<0.0001
	C18:1 *trans* isomers (mg/100 g fresh muscle)				
C18:1 *trans*-4	0.57	0.08	0.56	0.08	0.9219
C18:1 *trans*-5	0.53	0.07	0.47	0.07	0.5512
C18:1 *trans*-6/7/8[a]	2.94	0.34	1.94	0.34	0.0473
C18:1 *trans*-9	7.84	1.00	5.93	1.00	0.1885
C18:1 *trans*-10	24.05	3.31	6.85	3.31	0.0009
C18:1 *trans*-11	66.21	15.81	101.7	15.81	0.1233
C18:1 *trans*-12	24.99	3.81	20.25	3.81	0.3869
C18:1 *trans*-13/14[a]	20.04	6.14	37.64	6.14	0.0455
C18:1 *trans*-15	7.82	2.11	13.94	2.11	0.0487
C18:1 *trans*-16	8.22	2.51	15.71	2.51	0.0429

[a] Coeluation on GC.

C18:1 Trans Isomers of *Longissimus* Muscle. The proportion and concentration (mg/100 g fresh muscle) of the individual C18:1 *trans* isomers in the *longissimus* muscle are presented in **Table 4**. The separation of C18:1 *trans*-6, C18:1 *trans*-7, C18:1 *trans*-8, and C18:1 *trans*-13 and C18:1 *trans*-14 in beef and milk fat has not been achieved using gas chromatographic conditions (*28*, *29*).

The most abundant C18:1 *trans* isomer in the *longissimus* muscle was *trans*-vaccenic acid (C18:1 *trans*-11). The proportion of C18:1 *trans*-11 was significant by an increase in pasture-fed as compared with concentrate-fed bulls, 41.1 vs 49.4% of total C18:1 *trans*, respectively. Pasture feeding also caused significant variation in the distribution of the other C18:1 *trans*

isomers. The proportions of the isomers C18:1 *trans*-5, C18:1 *trans*-6−8, C18:1 *trans*-9, and C18:1 *trans*-10 decreased while the isomers C18:1 *trans*-12, C18:1 trans13−14, C18:1 *trans*-15, and C18:1 *trans*-16 increased. The concentration of C18:1 *trans*-11 tended to be higher in pasture-fed as compared to concentrate-fed bulls (102 vs 66.2 mg/100 g fresh muscle), but the values did not reach statistical significance ($p = 0.12$). In contrast, the concentration of C18:1 *trans*-13/14, C18:1 *trans*-15, and C18:1 *trans*-16 isomers was significantly higher in pasture-fed as compared with concentrate-fed bulls. The diet had no effect on the concentration of the isomers C18:1 *trans*-4, C18:1 *trans*-5, and C18:1 *trans*-9.

DISCUSSION

Fatty Acids in Triacylglycerides and PL. It is recognized that feeding diets with high proportions of *n*-3 long chain PUFA such as grass, although very low in total fat content, and fish oil (fat high in C18:3 *n*-3 and the *n*-3 long chain PUFA, respectively) increase the level of PUFA in the meat (*10*, *30*−*32*). The results in this study show that diet type affected the fatty acid composition of the PL and TG fractions of *longissimus* muscle.

The high content of linolenic acid in the lipids of grass and wilted silage, typically 50−65% of total fatty acids, is partly deposited into the muscle lipids despite the high levels of biohydrogenation of dietary PUFA in the rumen. The PLs and TGs of pasture-fed bulls contained significantly higher amounts of total *n*-3 fatty acids as compared with those fed on concentrates. This supports the concept that feeding diets rich in the building block of the *n*-3 PUFA series, C18:3 *n*-3, will increase the synthesis of the longer chain *n*-3 PUFA via chain elongation and desaturation, which is supported by other studies reported by Itoh et al. (*33*) and Lorenz et al. (*9*) for pasture-fed Simmental steers and bulls, respectively.

The essential fatty acids, C18:3 *n*-3 and C18:2 *n*-6, are compounds using the same enzymes for elongation and desaturation processes. These competitive metabolic interactions between *n*-3 and *n*-6 fatty acids in tissues are well-established (*34*). The proportions of total *n*-6 fatty acids in the PL were significantly decreased in the pasture-fed bulls, but no diet effect was observed in concentration (mg/100 g fresh muscle) of C18:2 *n*-6, C20:4 *n*-6, and total *n*-6 fatty acids (**Table 1**). Earlier investigations observed decreasing *n*-6 fatty acid contents and partially a greater decline in *n*-6 fatty acids in total *longissimus* muscle lipids on pasture-fed animals, e.g., Hereford × Friesian cattle (*6*), German Holstein steers (*11*), and German Simmental bulls (*9*). French et al. (*10*, *35*) reported a lower increase of *n*-3 fatty acids in the muscle of 85 day grazing continental crossbred steers and no diet effect on *n*-6 fatty acid contents in i.m. fat. Recently, Horrobin et al. (*36*) reported that a lower intake of *n*-3 fatty acids (C20:5 *n*-3) led to elevated concentrations of C20:4 *n*-6 in red blood cells of rats and humans. The authors suggested that collaboration and not the competitive metabolic interactions between the fatty acids C20:5 *n*-3 and C20:4 *n*-6 is the key to understanding biological pathways. According to our results, pasture feeding caused an accumulation of *n*-3 fatty acids and a significant decline of *n*-6 fatty acids in the total lipid (*24*) and in the triglyceride fraction of *longissimus* muscle, but no diet effect was detected for the *n*-6 fatty acids in the PL. This contributed toward very favorable *n*-6/*n*-3 ratios in both the PL and the TG fractions (**Tables 1** and **2**), which were lower than the target ratio of 5:1 recommended by the German Society of Nutrition (*12*). The long chain *n*-3 fatty acids (C20:5 *n*-3, C22:5 *n*-3, and C22:6 *n*-3) are mainly incorporated in the phospholipid fraction of the muscles as compared with the triglyceride fraction. Diet had a greater effect on the fatty acid composition of phospholipid relative to the triglyceride fraction. The most dominant fatty acids in the triglyceride fraction were oleic acid (C18:1 *cis*-9), palmitic (C16:0), and stearic acid (C18:0) (**Table 2**). The concentration of total saturated fatty acids tended to be lower in pasture-fed relative to concentrate-fed bulls. Lorenz et al. (*9*) found that proportions for C18:0 in the TG of the *longissimus* muscle of German Simmental bulls were not different on pasture vs concentrate feeding. Previous studies have also demonstrated beneficial reductions in amounts of myristic and palmitic acid in the TG of crossbred steers (*35*) and Angus and Simmental steers (*33*) fed on pasture. The consumption of saturated fatty acids (C14:0 and C16:0) has been associated with a rise of serum low-density lipoprotein cholesterol concentration to enhance the risk for coronary heart disease (*37*). In contrast, stearic acid has been shown to be neutral in its effect on plasma cholesterol in humans (*38*). Strategies that decrease the content of saturated fatty acids in i.m. fat of beef would improve the healthiness of beef and contribute to better consumer acceptance of the product.

CLA and *trans*-Vaccenic Acid Isomer Profiles. Many potentially beneficial health effects in animals and cell culture have been ascribed to CLAs when consumed as a mixture. The investigations generally focused on two isomers CLA *cis*-9,*trans*-11 and CLA *trans*-10,*cis*-12 (*14*, *15*). Studies of pure single isomers showed that they are different in biological activities as reported in current reviews by Banni et al. (*16*) and Martin et al. (*17*). In this investigation, the proportion of the main CLA isomer, CLA *cis*-9,*trans*-11 (determined by GC) was significantly increased from 0.50 to 0.74% of total fatty acids in concentrate- and pasture-fed bulls, respectively, as reported by Nuernberg et al. (*24*) and Dannenberger et al. (*39*). However, because of coelution, the values measured by GC included CLA *cis*-9,*trans*-11, CLA *trans*-7,*cis*-9, and CLA *trans*-8,*cis*-10. However, no diet effect was observed for CLA *cis*-9,*trans*-11 (including CLA *trans*-7,*cis*-9 and CLA *trans*-8,*cis*-10) on the absolute content basis (concentrate, 17.11 mg/100 g fresh muscle; pasture, 17.34 mg/100 g fresh muscle) (*24*). The CLA *cis*-9,*trans*-11 isomer can be formed in the rumen as an intermediate of biohydrogenation of linoleic to stearic acid. It was hypothesized that increasing the supply of linoleic acid would increase the amount of CLA *cis*-9,*trans*-11 synthesized in the rumen, thus available for incorporation into meat (*40*−*44*). Contrasting effects of high linoleic acid diets on tissue fatty acid composition in beef may be due to breed, and sex type was reported by Zembayashi et al. (*45*). Recently, Beaulieu et al. (*42*) observed increased total C18:1 *trans* concentrations in tissues of Angus-Wagyu heifers supplementing a high-corn diet with soybean oil; however, CLA *cis*-9,*trans*-11 content was highest in the subcutaneous fat but not affected in any other tissue by soybean oil supplement with 2.5 to 5.0%, a rich source of linoleic acid. Madron et al. (*43*) found significantly higher proportions of CLA *cis*-9,*trans*-11 in the intramuscular, intermuscular, and subcutaneous fat in crossbred Angus steers fed on diets containing 12.7−25.6% extruded full-fat soybeans, but the differences were relatively small. However, diets containing a proportionally high level of linolenic acid in the fat, such as fresh grass, grass silage, concentrates containing linseed, and pasture feeding with finishing periods, resulted in an increased deposition of CLA *cis*-9,*trans*-11 in the muscles (*10*, *30*, *46*−*49*). French et al. (*10*) reported a significant increase in CLA *cis*-9,*trans*-11 in muscle of crossbred steers grazed for 85 days on pasture as compared to concentrate, averaging 1.08 and

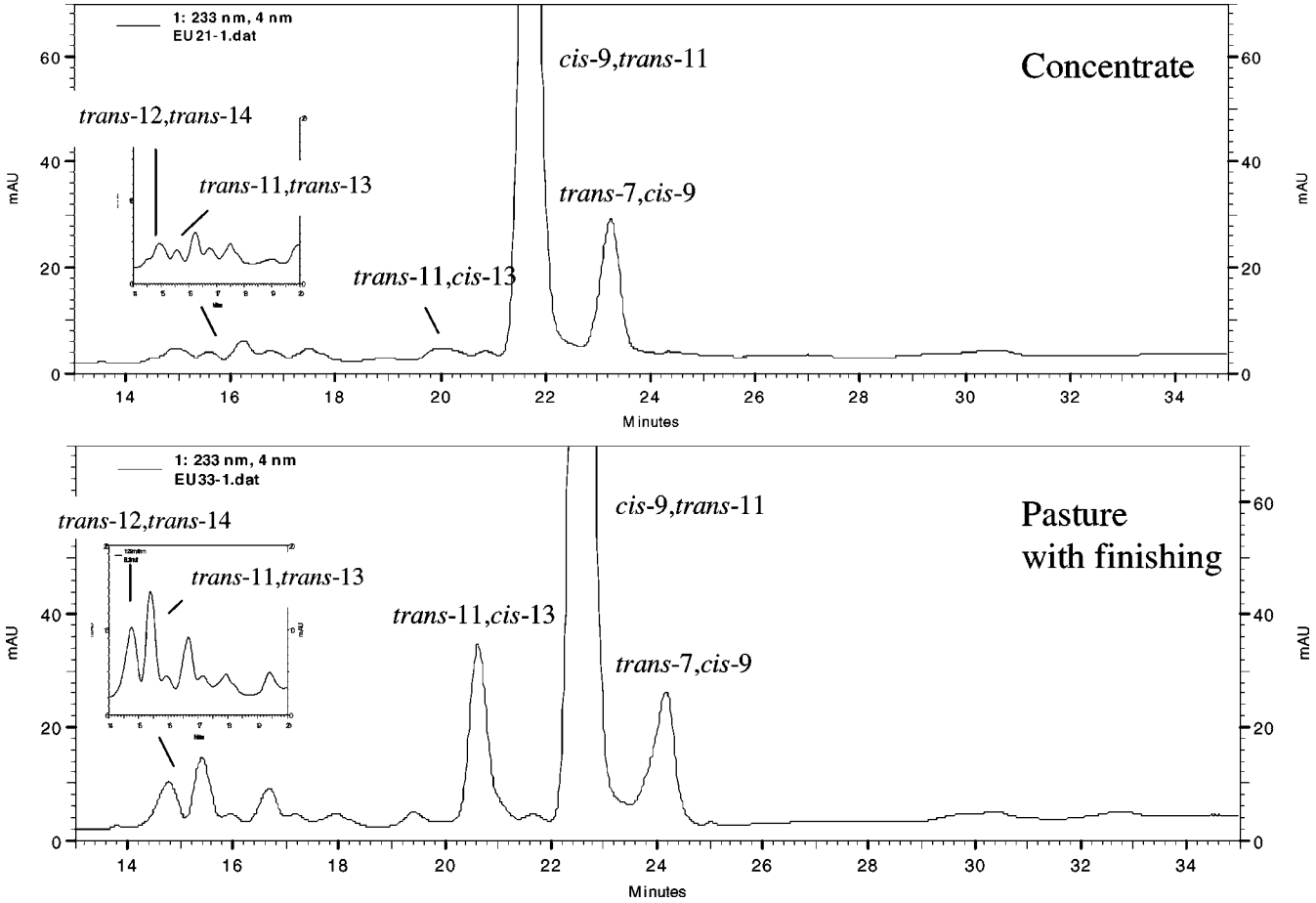

Figure 1. Ag^+-HPLC chromatograms of *longissimus* muscle of concentrate- and pasture-fed German Holstein bulls detected at 233 nm.

0.37% of total fatty acids, respectively. Dhiman et al. (*48*) and Poulson (*49*) observed a significant increase of CLA *cis*-9,*trans*-11 in the muscle of crossbred steers on forage and pasture without grain supplementation. Similarly, Steen and Porter (*50*) reported that the content of CLA *cis*-9,*trans*-11 in muscle and subcutaneous fat from steers finished on pasture was three times higher than in those on concentrate. However, biohydrogenation of C18:3 *n*-3 by rumen microorganisms does not include CLA *cis*-9,*trans*-11 as an intermediate (*40*). C18:1 *trans*-11 is formed during the biohydrogenation of linolenic acid to stearic acid; therefore, *trans*-vaccenic acid is a common intermediate in the metabolic pathway of linoleic and linolenic acid. Griinari and Bauman (*51*) concluded that a relatively small proportion of CLA *cis*-9,*trans*-11 formed in the rumen escapes and is available for deposition in the muscles. Therefore, the conversion of C18:2 *n*-6 to CLA *cis*-9,*trans*-11 by ruminal microorganisms does not appear to be the major source of CLA *cis*-9,*trans*-11 in meat (*51*). However, Griinari et al. (*22*) showed that the primary source of CLA *cis*-9,*trans*-11 was from the endogenous synthesis involving Δ^9-desaturase and *trans*-vaccenic acid. The infusion of C18:1 *trans*-11 postruminally, and therefore not available for modification by rumen microorganisms, increased the proportion of CLA *cis*-9,*trans*-11 in milk fat (*22*). Recently, Kay et al. (*52*) demonstrated that approximately 90% of CLA *cis*-9,*trans*-11 in milk fat was produced endogenously in cows fed fresh pasture. The results of our experiment showed that the C18:1 *trans*-11 was the most abundant C18:1 *trans* isomer in the *longissimus* muscle and the proportion of C18:1 *trans*-11 was increased in pasture as compared with concentrate-fed bulls from 41.1 to 49.4% of total C18:1 *trans*. However, the concentration of C18:1 *trans*-11, although numercially higher on pasture as compared to concentrate, was not significantly different (**Table 4**). Nevertheless, there was a correlation between individual C18:1 *trans* isomers and CLA *cis*-9,*trans*-11 in the muscle (based on absolute contents) as reported previously (*28*, *43*, *47*). In our study, the amount of CLA *cis*-9,*trans*-11 was strongly correlated ($p < 0.001$) with C18:1 *trans*-9 ($r = 0.75$, confidence interval 0.40−0.91), C18:1 *trans*-10 ($r = 0.86$, confidence interval 0.63−0.95), and C18:1 *trans*-11 ($r = 0.80$, confidence interval 0.50−0.93) in pasture as compared with the concentrate-fed bulls [C18:1 *trans*-9 ($r = 0.70$, confidence interval 0.32−0.89), C18:1 *trans*-10 ($r = 0.41$, not significant), and C18:1 *trans*-11 ($r = 0.62$, confidence interval 0.18−0.85)]. Surprisingly, the strongest correlation of CLA *cis*-9,*trans*-11 was found to C18:1 *trans*-10 in pasture-fed bulls, but no significant correlation to C18:1 *trans*-10 was found in concentrate-fed bulls. The other important parameter for the endogenous synthesis of CLA *cis*-9,*trans*-11 is the Δ^9-desaturase. The Δ^9-desaturase activity calculated as a Δ^9-desaturase index according to Malau-Aduli (*53*, *54*) and Kazala (*55*) was significantly decreased by pasture feeding in the *longissimus* muscle (concentrate, 72.9, vs pasture, 65.1). These facts may explain that namely the proportion of CLA *cis*-9,*trans*-11 was increased in the muscle of pasture as compared with concentrate bulls (0.50 vs 0.74 of total fatty acids), but the concentrations of CLA *cis*-9,*trans*-11 were unchanged between the different diets (concentrate, 17.11 mg/100 g fresh muscle; pasture, 17.34 mg/100 g fresh muscle) recently reported by Nuernberg et al. (*24*).

The results of the distribution of individual CLA isomers in the *longissimus* muscle showed that diets significantly affected the proportion of the individual CLA isomers. Using a double

Ag^+ HPLC column system, the proportion of CLA *cis*-9,*trans*-11 was significantly decreased from 73.5 to 65.0% of total CLA in concentrate- and pasture-fed bulls, respectively. Using Ag^+ HPLC, similar results with a decline of CLA *cis*-9,*trans*-11 proportions in *longissimus* muscle of German Simmental bulls from 77.0% of total CLA for concentrate to 66.5% of total CLA for pasture were reported by Nuernberg et al. (*11*). On Ag^+ HPLC-based investigations, Yurawecz et al. (*26*) found 74.8% CLA *cis*-9,*trans*-11 in a United States retail beef sample. Fritsche et al. (*21*, *28*) obtained 74−78% CLA *cis*-9,*trans*-11 in *longissimus* muscle from Charolais steers fed 30% silage and 70% concentrate. Previous studies showed that the isomer CLA *trans*-7,*cis*-9 is the second most abundant CLA isomer in beef meat and milk products (*21*, *28*, *29*, *56*−*58*). In our investigations, we found that the CLA isomer distribution was affected by the diet. In the concentrate group, CLA *trans*-7,*cis*-9 was the second most abundant CLA isomer (9.4% of total CLA). Pasture feeding significantly decreased CLA *trans*-7,*cis*-9 to 6.0% of total CLA. In the pasture-fed bulls, another isomer, CLA *trans*-11,*cis*-13, was significantly increased up to 12.4% of total CLA as compared with concentrate-fed bulls (**Table 3**). Recently, Nuernberg et al. (*11*) and Kraft et al. (*59*) found a similar effect in *longissimus* muscle of grazing German Simmental bulls and in milk fat from grazing cows in the Alps (Switzerland), respectively. The partial chromatograms in **Figure 1** show the main differences in CLA isomer distribution of concentrate-fed as compared with pasture fed bulls.

Kraft et al. (*59*) discussed that plants growing under lower temperatures contain lipids with a higher percentage of linolenic acid. C18:3 *n*-3 has been shown to be converted to C18:3 *cis*-9,*trans*-11,*cis*-15 conjugated trienes in the rumen (*40*). The next step is the hydrogenation to C18:2 *trans*-11,*cis*-15, which undergoes further hydrogenation, depending on the bacteria involved, leaving *trans*-vaccenic acid. The final product is stearic acid (*40*). Kraft et al. (*59*) discussed that the *trans*-11 double bond seems to be the most stable *trans* bond among the C18:1 and CLA isomers during the ruminal fermentation and observed larger amounts of three of the possible CLA isomers with a *trans*-11 double bond, CLA *cis*-9,*trans*-11, CLA *trans*-11,*cis*-13, and CLA *trans*-11,*trans*-13 in milk from grazing cows in the Alps. Our investigations confirm these results. We found that among the CLA *trans*,*trans* isomers, the CLA *trans*-11,*trans*-13 was the most abundant compound up to 5.6% followed by the CLA *trans*-12,*trans*-14 (3.0%) and CLA *trans*-9,*trans*-11 (2.2%) of total CLA in pasture-fed bulls (**Table 4**).

In conclusion, pasture feeding enhanced the absolute *n*-3 fatty acid concentrations in phospholipid and triglyceride fraction of muscle lipids. The important nutritional value, the *n*-6/*n*-3 ratio, was beneficially decreased to lower than <5:1, hence achieving an important target with respect to human health. The concentration of CLA *cis*-9,*trans*-11 in muscle lipids was not influenced significantly by different diets. Beef enriched with *n*-3 fatty acids by pasture feeding represents a source of *n*-3 fatty acid and CLA intake for humans. Future investigations should focus on enhancing CLA *cis*-9,*trans*-11 contents in the muscles and include variations of dietary conditions to increase the rumen production of *trans*-vaccenic acid and the activity of Δ^9-desaturase. More experiments are necessary to explain the differences in the CLA isomer distribution in the muscles between the concentrate-fed and the pasture-fed ruminants.

ABBREVIATIONS USED

CLA, conjugated linoleic acids; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; GC, gas chromatography; PL, phospholipids; TG, triglycerides; w, weight; v, volume; LSM, least squares mean; SE, standard error of LSM; ME, methylester; FAME, fatty acid methyl ester; i.m. fat, intramuscular fat.

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