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Relationship between Lipid Peroxidation and Antioxidant Status in the Muscle of German Holstein Bulls Fed *n*-3 and *n*-6 PUFA-Enriched Diets

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This study evaluated the influence of different *n*-3 and *n*-6 PUFA-enriched diets on the relationship between lipid peroxidation and antioxidant status by analyzing fatty acids (FA), lipid peroxidation, antioxidant capacity (AOC), antioxidant enzymes, trace elements, and fat-soluble vitamins in the longissimus muscle. Diet caused significant changes in muscle FA composition, leading to accumulation of beneficial *n*-3 FA. β-Carotene and catalase activity were significantly elevated in muscle of the *n*-3 PUFA-enriched diet group compared to the *n*-6 PUFA-enriched diet group. Lipid peroxidation was higher in muscle of the *n*-3 PUFA-enriched diet group after 15 min of reaction time. There was no significant effect of diet on AOC, but it increased with reaction time. The present results suggest that the antioxidant defense in muscle of the *n*-3 PUFA-enriched diet group could balance reactive substances under low oxidative conditions. However, the antioxidant capacity was not sufficient under abundant accumulation of reactive substances.

KEYWORDS: Antioxidants; beef; endogenous enzymes; TBARS; vitamins

INTRODUCTION

High consumption of saturated fatty acids (SFA) raises plasma low-density lipoprotein (LDL)-cholesterol, which is a major risk factor for arteriosclerosis and coronary heart disease (CHD). In contrast, selected monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) have a number of health benefits (*1*). PUFA have been shown to protect against CHD by lowering plasma LDL-cholesterol. A majority of the health benefits are associated with *n*-3 PUFA (*2*). A variety of PUFA differing in their chemical structures, such as *n*-6 and *n*-3 fatty acids (FA), play essential roles in many biological functions. In ruminants, the dietary FA are extensively metabolized and biohydrogenated in the rumen, resulting in a broad range of MUFA, PUFA isomers, and SFA. The MUFA intermediates are transformed to longer chain PUFA in muscle by lipogenic enzymes, for example, stearoyl-CoA-desaturase (SCD), Δ6-desaturase (Δ6d), and Δ5-desaturase (Δ5d). Diet has been considered the primary way to manipulate the lipid composition in beef tissues (*3*). However, increased intake of *n*-6 PUFA may be detrimental because *n*-6 PUFA are the precursors of pro-inflammatory eicosanoids. In contrast, increased intake of *n*-3 PUFA and a low *n*-6/*n*-3 FA ratio (< 4:1) are believed to be beneficial for various aspects of human health (*4*). Many studies have been conducted for the purpose of accumulating *n*-3 PUFA in beef (*3*, *5*, *6*). Additional interest has been focused on the preservation of *n*-3 PUFA for human consumption because these PUFA are highly susceptible to lipid peroxidation by highly reactive species originating from endogenous and exogenous sources. Increasing the degree of unsaturation in muscle tissue increases its susceptibility to lipid peroxidation, which is considered to be the major cause of meat quality deterioration, affecting color, flavor, and nutritional value (*7*). In addition to FA composition, other factors influence the susceptibility of meat to lipid peroxidation (*8*); specifically, an imbalance of antioxidant defenses to oxidative processes is the predominant factor that increases lipid peroxidation (*9*). Yet there is a lack of investigations regarding the relationship between lipid peroxidation and antioxidant status in beef from animals fed diets enriched in PUFA (*10*, *11*). In beef cattle, most research on this topic has focused on stabilizing PUFA with antioxidant supplements (*12*−*14*) or centered around the effect of different basal diets (pasture, grass silage, maize silage) sans PUFA enrichment (*15*, *9*, *16*). Therefore, we designed the present study to investigate the effects of *n*-3 and *n*-6 PUFA-enriched diets on the mechanisms that regulate FA biosynthesis in various beef tissues from German Holstein bulls fed maize silage with soybean-based concentrate and grass silage with linseed oil and rapeseed cake-supplemented concentrate. In particular, our objective was to study the influence of different *n*-3 and *n*-6 PUFA-enriched diets on the relationship between lipid

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Table 1. Chemical and Fatty Acid Composition (Grams per 100 g) of Total Mixed Ratio (TMR)

	TMR for control group	TMR for exptl group
chemical composition		
metabolic energy (MJ/kg)	11.4	11.1
crude protein	15.3	14.9
crude fat	3.1	4.0
crude ash	7.0	12.3
fatty acid profile		
C12:0	0.2	0.2
C14:0	0.8	0.4
C16:0	20.5	16.7
C18:0	2.6	2.6
C18:1*cis*-9	19.1	15.7
C18:2*n*-6	40.0	21.5
C18:3*n*-3	10.8	35.5
n-6/*n*-3 fatty acid ratio	3.7	0.6
lipid-soluble vitamins (mg/kg)[a]		
retinol (vitamin A)	13.2	14.9
α-tocopherol	265.0	252.5
δ-tocopherol	2.7	nd[b]
γ-tocopherol	42.6	24.6
β-carotene	165.0	999.0
trace elements (mg/kg)[a]		
Fe	208.3	720.0
Cu	20.0	33.1
Zn	131.5	168.0
Se	0.7	1.0

[a] Based on fresh material. [b] nd, not detected.

peroxidation and antioxidant status by analyzing FA, lipid peroxidation, total antioxidant capacity (AOC), antioxidant enzymes, trace elements, and lipid-soluble vitamins in the longissimus muscle.

MATERIALS AND METHODS

Animals and Diets. A description of experimental animals and conditions has already been published for a subgroup of animals by Herdman et al. (*17*). The results presented in this paper were obtained from a total of 29 German Holstein bulls that were randomly selected and assigned one of the test diets: a control diet ($n = 15$) containing maize silage supplemented with concentrate enriched with *n*-6 FA or an experimental diet ($n = 14$) containing grass silage supplemented with concentrate enriched with *n*-3 FA (**Table 1**). Bulls were slaughtered when reaching 623−630 kg liveweight by captive bolt stunning followed by exsanguination in the abattoir of the Leibniz Institute for Farm Animal Biology in Dummerstorf (Germany). The slaughter and dressing procedures were in accordance with European Union specifications. Longissimus muscles were taken immediately after slaughter to test for thiobarbituric acid reactive substances (TBARS), enzyme activities, and AOC and stored at −70 °C until the respective analysis. Likewise, after 24 h of chilling, samples were for the determination of FA as well as vitamin and trace element content and stored at −20 °C. All samples were taken from the 6th−13th rib of the right carcass side.

Fatty Acid Composition. Lipids from 2 g of muscle were extracted with chloroform/methanol (2:1 v/v) according to the method of Folch et al. (*18*) by homogenization (Ultraturrax, 3 × 15 s, 12000 rpm) at room temperature. The FA composition of muscle lipids and feed was determined using the methodology described by Nuernberg et al. (*19*). Briefly, the methodology involved capillary gas chromatography (GC) on a CP SIL 88, 100 m × 0.25 mm × 0.25 μm capillary column (Chrompack, Varian, USA) installed in a Perkin-Elmer gas chromatograph Autosys XL with a flame ionization detector and split injection. The temperature program for the muscle FA was 150 °C, held for 5 min, increased to 200 °C at a rate of 2 °C min^{-1}, held for 10 min, and then increased to 220 at 1.5 °C min^{-1}, and held for 35 min. Hydrogen was used as the carrier gas at a flow rate of 1 mL min^{-1}. The split ratio was 1:40, the injector temperature was set at 260 °C and the detector at 280 °C. 19:0 methyl ester was used as internal standard.

Lipid Peroxidation. To assess the amount of lipid peroxidation, a TBARS assay was used according to the methodology recently described by Mahecha et al. (*6*). To stimulate lipid peroxidation, 3 mL of the muscle homogenate was incubated with 0.1 mM ascorbate and 5 μM $FeSO_4$. From this, 0.5 mL was immediately removed and pipetted into 0.25 mL of 20% trichloric acid (TCA) in 100 mM KCl. The remaining incubated homogenate was placed in a water bath of 37 °C, and after 0, 15, 30, 60, and 120 min, 0.5 mL of this incubated homogenate was pipetted into the TCA medium (see above). These samples were centrifuged at 10000*g* for 10 min, and 0.5 mL of the supernatants was mixed with 0.5 mL of thiobarbituric acid (0.67%) and boiled for 15 min in a water bath. After immediate cooling, the absorbance at 535 nm was determined. Standard malondialdehyde (MDA) solution was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane, and the results were expressed as nM per g of muscle.

AOC. The AOC of muscle samples was determined using two different assays: ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC). Details of the methodologies were recently described by Mahecha et al. (*20*). Sample preparation for both assays were carried out using a hydrophilic (distilled water) and a lipophilic extraction solvent system (acetone/ethanol/distilled water, 5:4:1 v/v/v) to encompass AOC of both lipophilic and hydrophilic antioxidants in the muscle. As such, the methods are called $FRAP_{water}$, $FRAP_{lipid}$, $TEAC_{water}$, and $TEAC_{lipid}$ assays. Briefly, approximately 5 g of frozen muscle sample was minced by the use of a kitchen disintegrator, mixed with 5 mL of the respective extraction solvent, and homogenized with an Ultra Turrax (IKA Labortechnik, Staufen, Germany). Samples were centrifuged at 20414*g* and 4 °C for 30 min (Allegra 21R, Beckman Coulter, Krefeld, Germany). Extracts were filtered and used for immediate analysis. Measurements were carried out in triplicate.

FRAP Assay. FRAP reagent solution consisted of 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,4-tri(2-pyridyl)-*s*-triazine (TPTZ) in 40 mmol/L hydrochloric acid, and 20 mmol/L iron(III) chloride hexahydrate at the ratio of 10:1:1. A freshly prepared reagent solution (2950 μL) was mixed with 50 μL of the muscle extract. Extinction was measured at 593 nm (UV−vis spectrophotometer UV-2401 PC, Shimadzu, Japan) at 5, 30, and 60 min after the addition of the reagent solution to the muscle extract. The amount of reduced iron was determined by calibration of the method using known concentrations of iron(II) sulfate-7-hydrate between 0.2 and 3.9 mmol/L Fe(II). FRAP antioxidant capacity was expressed as Fe^{2+} equivalents in micromoles per gram of muscle.

TEAC Assay. To generate the ABTS radical, a stock solution of 8 mmol/L 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and 3 mmol/L $K_2S_2O_8$ (potassium peroxodisulfate) was prepared by dissolution in water and incubation in the dark overnight at room temperature. Prior to use, the reagent solution was diluted in phosphate-buffered saline (PBS buffer, pH 7.4) or methanol, respectively, to an absorbance of 1.3 at a wavelength of 734 nm. The ABTS reagent solution (2950 μL) was mixed with 50 μL of the muscle extract, and the decrease of absorbance was measured after 5, 30, and 60 min of reaction time. AOC was determined by calibration using known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) between 0.08 and 1.9 mmol/L. TEAC AOC was expressed as Trolox equivalents in micromoles per gram of muscle. Both analyses were carried out using a UV−vis recording spectrophotometer (UV-2401 PC, Shimadzu).

Endogenous Enzymes. The activity of antioxidant enzymes was measured as previously described (*20*). The sample preparation started with frozen samples, and during the whole preparation procedure the extracts were kept on an ice bath to prevent enzymatic reaction and oxidation of unsaturated FA. The samples were analyzed twice with one replicate. The tissue was homogenized, and 5 g of muscle was mixed with 10 mL of ice-cold phosphate buffer (extraction solvent, pH 7.0, 50 mM; disodium phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and KH_2PO_4). Samples were homogenized by UltraTurrax (13000 rpm, ca. 45 s). After centrifugation (7000 rpm, 20 min, 4 °C), the supernatants were recovered and filtered over glass wool.

Catalase (*CAT*). CAT activity was measured according to the procedures of Aebi (*21*) by following the rate of disappearance of hydrogen peroxide (H_2O_2). Fifty microliters of the muscle extracts was brought in a quartz cuvette (1 cm path length), and 2.9 mL of H_2O_2 solution was added.

Immediately, the absorbance was monitored at 240 nm during 100 s using a UV−vis spectrophotometer (UV-2401 PC, Shimadzu). CAT activity was expressed in micromoles per minute per gram (U/g). One unit (U) of CAT activity was defined as the amount of extract needed to decompose 1 μmol of H_2O_2 per minute.

Superoxide Dismutase (*SOD*). SOD activity was assayed by the methodology of Marklund and Marklund (*22*) by following the inhibition of 1,2,3-trihydroxybenzene (pyrogallol) autoxidation. The same muscle extract obtained for CAT was used to measure SOD activity. Six different amounts of muscle extracts were prepared for each sample, 0, 10, 20, 30, 40, and 50 μL of extract, by adding 175, 165, 155, 145, 135, and 125 μL of distilled water, respectively. In each cuvette, 2.75 mL of Tris−cacodylic buffer (pH 8.2, 50 mM with diethylenetriaminepentaacetic acid (DTPA), cacodylic acid, and 75 μL of pyrogallol solution (15 mM)) was added to start the reaction. The absorbance was monitored at 420 nm during 300 s using a UV−vis spectrophotometer (UV-2401 PC, Shimadzu). A linear regression curve was fit, and the equation was used to determine the amount of extract needed to inhibit the reaction by 50%. The result was expressed as units per gram of sample. One unit was taken as the SOD activity that inhibits the reaction by 50%.

Glutathione Peroxidase (*GSH-Px*). GSH-Px was measured by following GSH reduction coupled to NADPH oxidation by glutathione reductase (*23*). Muscle extracts were obtained following the same methodology described above for CAT. Three hundred microliters of the muscle extracts, 2.65 mL of reaction solvent (1.13 mM reduced glutathione; 0.57 mM ethylenediaminetetraacetic acid (EDTA), 1.13 mM NaN_3, and 190 μL of glutathione reductase dissolved in 100 mL of cold phosphate buffer), 26 μL of NADPH solution (17.3 mM), and 20 μL of H_2O_2 solution (22.5 mM) were given in cuvettes (1 cm path length). The absorbance was monitored at 340 nm during 400 s using a UV−vis spectrophotometer (UV-2401 PC, Shimadzu). GSH-Px activity was expressed as micromoles of oxidized NADPH per microliter per minute per gram (U/g). One unit of GSH-Px activity was defined as the amount of extract required to oxidize 1 μmol of NADPH per minute at 22 °C.

Fat-Soluble Vitamins. Retinol (vitamin A), tocopherol isomers, and β-carotene were extracted according to the methodology recently described in detail by Mahecha et al. (*6*). Briefly, three subsamples were prepared by homogenizing tissue (4 g per subsample) in 6 mL of a mixture composed by 0.15 M potassium chloride and 0.05 M Tris buffer, using an Ultraturrax (3 × 15 s, 34000 rpm; at room temperature). The tubes were introduced in a water bath (70 °C) for 5 min. Nitrogen was added again for 10 s, followed by the addition of 5 mL of potassium hydroxide solution (10 N, pH 7.4). The tubes were again heated in a water bath (70 °C) for 30 min. After cooling, the mixture was extracted four times with 4 mL of *n*-hexane/ethyl acetate 2:1 (v/v with 0.01% butylhydroxytoluene (BHT)). All samples were analyzed using an HPLC system (Shimadzu LC-10 AD) equipped with a Sil-10A Shimadzu automatic injector, SPD-10 AV, UV−V15 spectrophotometer Shimadzu detector (for retinol (325 nm) and β-carotene (454 nm)), RF-10A Shimadzu spectrophotometer detector (for α-, γ-, δ-tocopherol; excitation, 295 nm; emission, 330 nm). Both detectors were used in series. Different vitamins were identified and quantified with the use of an external standard procedure. The external calibration plots were generated for a standard mixture in the range of approximately 5−120 μg/mL.

Trace Elements. The determination of selected trace element content including selenium, copper, iron, and zinc (Se, Cu, Fe, and Zn, respectively) was performed on muscle tissue using an inductively coupled plasma mass spectrometer (ICP-MS 7500ce, Agilent Technologies) as recently described (*6*, *24*). Briefly, after thawing, the samples were mixed, and approximately 1 g of tissue was treated with 2 mL of nitric acid (65%), 0.5 mL of hydrochloric acid (37%), and 2 mL of deionized water. The sample preparation was carried out using microwave-aided pressure disintegration (CEM, Kamp-Lintfort, Germany). Trace elements were analyzed twice for each sample and expressed as milligrams per kilogram of fresh muscle.

Color. Lightness (L^*), redness (a^*), and yellowness (b^*) coordinates (CIELAB color system) were measured in samples after 24 h using a Konika Minolta Chromameter CR 300 (Japan) according to the methodology described by Nuernberg et al. (*25*). Color was measured at three positions on one sample.

Reagents. For FA analysis a reference standard "Sigma-FAME mixture" was obtained from Sigma-Aldrich (Deisenhofen, Germany). Additionally, individual methyl esters of 18:4 *n*-3, 22:4 *n*-6, and 22:5 *n*-3 were purchased from Matreya (Pleasant Gap, PA). Methyl esters of 18:1*trans*-11 and 18:1*cis*-11 were purchased from Larodan Fine Chemicals (Malmö, Sweden). All solvents used for GC and HPLC were of HPLC grade from Lab-Scan (Dublin, Ireland). Reagents for the AOC assays TPTZ (≥99%), ABTS, and Trolox were obtained from Fluka (Buchs, Switzerland) or from Sigma-Aldrich. Reagents for endogenous enzyme activity investigations DTPA, cacodylic acid, reduced glutathione, NaN_3, glutathione reductase, and NADPH were purchased from Sigma-Aldrich. All inorganic compounds used for buffer and solutions were obtained from C. Roth (Karlsruhe, Germany).

Statistical Analysis. All data were analyzed by the least-squares method using GLM procedures of SAS. The following model was used for most traits: $Y_i = \mu + D_i + E_{ij}$, where Y_i represents an observation, μ is the overall mean, D_i is the effect of *i*th diet ($i = 1, 2$), and E_{ij} is the residual error. For AOC, the repeated effect of reaction time and the interaction of diet × reaction time were also evaluated. All tables contain the least-squares mean (LSM) and the standard error (SEM) of the LSM. All statistical tests of LSM were performed for a significance level of $p \leq 0.05$ using Tukey's test. Tendency was considered when $p \leq 0.10$. Relationships between different parameters were assessed after calculation of Person correlation coefficients.

RESULTS

Fatty Acid Composition. Diet caused significant changes in muscle FA composition. Treatment diet decreased the concentration of C14:0, C14:1, C16:0, C16:1*cis*-9, 18:1*cis*-9, C18:1*cis*-11, C18:1*trans*-10, the sum of MUFA, the sum of SFA, linoleic acid (C18:2*n*-6), *n*-6 FA, and the *n*-6:*n*-3 ratio. There was no effect on the sum of PUFA, CLA*cis*-9,*trans*-11, vaccenic acid (VA, C18:1*trans*-11), or on the sum of *trans*-FA. The concentrations of linolenic acid (C18:3*n*-3), eicosapentaenoic acid (EPA, C20:5*n*-3), docosapentaenoic acid (DPA, C22:5*n*-3), docosahexaenoic acid (DHA, C22:6 *n*-3), and *n*-3 FA increased significantly with treatment diet (**Table 2**).

Lipid Peroxidation. The effect of diet on lipid peroxidation was measured given that TBARS was dependent on the reaction time. The longissimus muscle of control and treatment animals exhibited similar lipid peroxidation at the starting point (23.4 and 23.9 mM MDA/g of sample, respectively). At 15 min, only a slight increase in lipid peroxidation was evident in muscle of treatment animals. However, diet considerably affected TBARS values at subsequent time points: 28, 33, and 23% higher at 30, 60, and 120 min, respectively (**Figure 1**).

Antioxidant Status. *Fat-Soluble Vitamins*. Diet showed different effects on lipid-soluble vitamins in muscle of German Holstein bulls. Whereas the concentration of β-carotene significantly increased in muscle of treatment animals, the concentration of α-tocopherol tended to decrease, and there was no effect on retinol (**Figure 2**). δ-Tocopherol did not change in treatment animals (0.003 vs 0.004 mg/kg of muscle, $p = 0.8$), whereas γ-tocopherol decreased significantly (0.078 vs 0.041, $p = 0.001$).

Endogenous Enzyme Activity and Trace Element Content. Muscle from treatment animals exhibited significantly higher CAT activity, almost twice that of control animals. GSH-Px activity was also higher in the muscle of the treatment group, but the difference was not statistically significant. SOD activities were similar for both groups (**Table 3**). Diet did not show significant effects in muscle for the trace elements iron, selenium, copper, and zinc (**Table 4**).

AOC. Two assays (FRAP and TEAC) carried out using both extraction systems (hydrophilic and lipophilic) showed no significant differences between the two different diet systems. Hydrophilic values were higher than lipophilic values according to both AOC assays. The $FRAP_{lipid}$ assay detected only trace amounts of antioxidants. AOC values increased significantly over time with

Table 2. Fatty Acid Composition (Milligrams per 100 g) of Longissimus Muscle from German Holstein Bulls Fed Different Diets

fatty acid	control group[a] (n = 15) LSM_{SEM}	exptl group[a] (n = 14) LSM_{SEM}
C12:0	$1.5_{0.1}$a	$1.0_{0.1}$b
C14:0	$63.4_{6.0}$a	$42.6_{6.3}$b
C14:1	$16.4_{1.9}$a	$10.9_{2.0}$b
C16:0	$627.3_{52.0}$a	$448.1_{53.9}$b
C16:1	$89.2_{8.1}$a	$57.9_{8.3}$b
C18:0	$342.6_{25.6}$	$277.4_{26.5}$
C18:1 *trans*-6−8	$3.7_{0.5}$	$2.5_{0.5}$
C18:1*trans*-9	$5.1_{0.5}$	$4.5_{0.5}$
C18:1 *trans*-10	$7.1_{1.0}$a	$4.1_{1.0}$b
C18:1*trans*-11	$13.6_{1.2}$	$13.9_{1.2}$
C18:1*cis*-9	$892.8_{76.3}$a	$614.9_{79.0}$b
C18:1*cis*-11	$28.8_{2.1}$a	$22.7_{2.2}$b
C18:2*n*-6	$112.9_{3.3}$a	$95.2_{3.4}$b
C18:3*n*-3	$13.0_{1.1}$a	$33.4_{1.1}$b
C20:4*n*-6	$29.9_{1.0}$a	$26.4_{1.0}$b
C20:5*n*-3	$3.8_{0.3}$a	$8.8_{0.3}$b
C22:5*n*-3	$8.4_{0.3}$a	$12.0_{0.3}$b
C22:6*n*-3	$1.0_{0.1}$a	$1.4_{0.1}$b
cis-9,*trans*-11 CLA[b]	$6.3_{0.7}$	$5.4_{0.7}$
Σ *trans*-FA	$30.3_{2.9}$	$25.2_{3.0}$
Σ SFA[c]	$1078.7_{84.8}$a	$805.5_{87.7}$b
Σ MUFA[d]	$1083.8_{97.1}$a	$752.0_{95.3}$b
Σ PUFA[e]	$187.8_{5.6}$	$191.9_{5.8}$
Σ *n*-3 FA[f]	$27.5_{1.4}$a	$56.5_{1.4}$b
Σ *n*-6 FA[g]	$157.6_{4.3}$a	$131.5_{4.4}$b
n-6/*n*-3	$5.8_{0.1}$a	$2.3_{0.1}$b

[a] Means with different letters (a, b) are significantly different at $p \leq 0.05$. [b] Coelution with C18:2 *trans*-7,*cis*-9 and C18:2 *trans*-8,*cis*-10. [c] The sum of saturated fatty acids (SFA) was calculated as 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. [d] The sum of monounsaturated fatty acids (MUFA) was calculated as C14:1 + C15:1 + C16:1 + C17:1 + Σ C18:1 *trans* + C18:1*cis*9 + C18:1*cis*-11 + C22:1 + C24:1. [e] The sum of trans fatty acids (PC18:1*trans*) was calculated as the sum of the isomers C18:1*trans*-6,*trans*-11. [f] Sum of *n*-3 fatty acids (FA) was calculated as the 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. [g] The sum of *n*-6 FA was calculated as the 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.

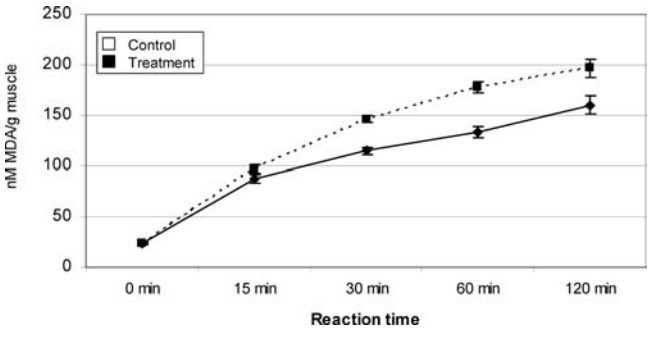

Figure 1. Lipid peroxidation of longissimus muscle from German Holstein bulls fed different diets as measured by TBARS at different times: at 0 min, p = 0.71; at 15 min, p = 0.09; at 30 min, p < 0.0001; at 60 min, p < 0.0001; at 120 min, p = 0.01.

all assays (**Table 5**). There was no significant relationship between diet and reaction time.

Relationship between Lipid Peroxidation and Antioxidant Status. Lipid peroxidation at 60 min had a slight positive, but significant, correlation to CAT activity (0.53; p = 0.003) and β-carotene concentration (0.45; p = 0.01); a slight tendency was noted to GSH-Px activity (0.34; p = 0.07). A negative slightly

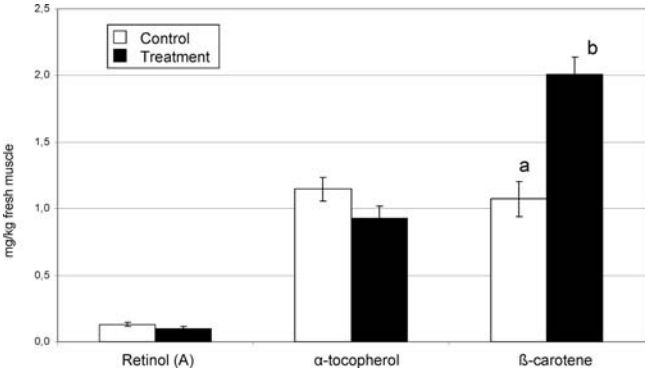

Figure 2. Vitamin concentrations of longissimus muscle from German Holstein bulls fed different diets: retinol (A), p = 0.12; α-tocopherol, p = 0.09; β-carotene, p < 0.0001. Means with different letters are significantly different at $p \leq 0.05$.

Table 3. Effect of Diet on the Activity of Endogenous Enzymes in Longissimus Muscle of German Holstein Bulls Fed Different Diets (Units /per Gram)

	control LSM_{SEM}	treatment LSM_{SEM}	p value
CAT	$45.5_{7.4}$	$79.7_{7.6}$	0.003
SOD	$5.6_{0.2}$	$5.4_{0.2}$	0.48
GSH-Px	$1.39_{0.1}$	$1.60_{0.1}$	0.25

Table 4. Selected Trace Element Concentrations and Color of Longissimus Muscle from German Holstein Bulls Fed Different Diets

	control LSM_{SEM}	treatment LSM_{SEM}	p value
trace elements (mg/kg of sample)			
Fe	$23.2_{1.12}$	$21.2_{1.20}$	0.2
Zn	$61.3_{2.10}$	$60.9_{2.17}$	0.9
Se	$0.16_{0.006}$	$0.15_{0.006}$	0.3
Cu	$1.21_{0.12}$	$1.18_{0.12}$	0.9
color			
L^*	$33.2_{0.5}$	$31.2_{0.5}$	0.01
a^*	$18.0_{0.4}$	$17.5_{0.4}$	0.3
b^*	$2.15_{0.3}$	$1.17_{0.3}$	0.03

significant correlation was found to α-tocopherol (−0.42; p = 0.02), and no correlation was detected to SOD.

Color. There was no effect of diet on redness. However, lightness and yellowness decreased in treatment animals (**Table 4**).

DISCUSSION

FA Composition. Beef muscle from treatment animals fed grass silage enriched with *n*-3 PUFA was clearly superior with regard to a more beneficial FA profile (higher linolenic acid, EPA, DHA, *n*-3 PUFA; lower *n*-6/*n*-3 ratio; and lower SFA) in comparison to control animals fed maize silage enriched with *n*-6 PUFA. Other studies have also found positive effects of dietary PUFA *n*-3 on muscle FA composition (*3*, *5*, *6*). The strong decrease in the *n*-6/*n*-3 ratio and a lower concentration of SFA, especially palmitic acid as was obtained in this work, are considered to be beneficial for human health (*2*, *4*). They could be associated with the high inclusion of *n*-3 PUFA in the treatment diet. Some studies report a general inhibition of de novo FA synthesis by unsaturated FA (*26*, *27*). However, in beef muscle, results from some studies using PUFA supplementation suggest a decrease (*28*), whereas others suggest an increase of de novo FA synthesis (*29*). Our results indicate a decrease in de novo synthesis (C12:0, C14:0, C16:0) by the treatment diet enriched with *n*-3 PUFA compared

Table 5. Effects of Diet and Reaction Time on the Total Antioxidant Capacity in Longissimus Muscle of German Holstein Bulls Fed Different Diets

	diet[a]		reaction time[a]			*p* value[b]		
	control LSM_{SEM}	treatment LSM_{SEM}	5 min LSM_{SEM}	30 min LSM_{SEM}	60 min LSM_{SEM}	D	T	D × T
$FRAP_{water}$[c]	$1.75_{0.09}$	$1.83_{0.1}$	$0.86_{0.07}$a	$1.98_{0.07}$b	$2.53_{0.07}$c	0.55	<0.0001	0.60
$FRAP_{lipid}$[c]	$0.38_{0.02}$	$0.35_{0.02}$	$0.24_{0.02}$a	$0.38_{0.02}$b	$0.48_{0.02}$c	0.49	<0.0001	0.55
$TEAC_{water}$[d]	$9.40_{0.19}$	$9.43_{0.20}$	$5.62_{0.14}$a	$10.23_{0.14}$b	$12.39_{0.14}$c	0.90	<0.0001	0.84
$TEAC_{lipid}$[d]	$3.71_{0.23}$	$3.46_{0.23}$	$1.63_{0.19}$a	$3.83_{0.19}$b	$5.30_{0.19}$c	0.44	<0.0001	0.87

[a] Means with different letters are significantly different at $p \leq 0.05$ (a−c for reaction time). [b] D, diet; T, time; D × T, interaction diet × time. [c] FRAP values are expressed as Fe^{II} equivalent in μmol/g of sample. [d] TEAC values are expressed as Trolox equivalents in μmol/g of sample.

to the control diet enriched with *n*-6 PUFA (**Table 2**). The increase of some FA from ruminal biohydrogenation, such as CLA*trans*-10,*cis*-12, and *trans* C18:1 isomers, is known to exert antilipogenic effects in bovine tissues (*30*, *31*). In the present study, the concentration of the sum of trans isomers did not change; however, the concentration of CLA*trans*-10,*cis*-12 significantly decreased with the treatment diet (0.25 vs 0.17 mg/100 g of muscle, for control and treatment animals, respectively). On the other hand, the concentration of CLA*cis*-9,*trans*-11 and its precursor VA did not increase with treatment diet; there was only a significant increase in the relative proportion of VA (0.6 vs 0.8 for control and treatment groups, respectively). Grazing animals on pasture, feeding them fresh forage, or increasing the amount of forage in their diet has been demonstrated to elevate the percentage of CLA*cis*-9,*trans*-11 in muscle from ruminants (*25*, *32*). However, supplementing these diets with C18:2- or C18:3-rich plant oils has yielded varied results. Herdman et al. (*17*) found a decrease in the stearoyl-coA desaturase (SCD) activity. This enzyme is involved not only in the conversion of VA to CLA*cis*- 9,*trans*-11 but also in the conversion of C16:0 and C14:0 into the corresponding MUFA C16:1*cis*-9 and C14:1*cis*-9 (*33*), in the conversion of C18:0 to C18:1*cis*-9, and in general, in the catalysis of the conversion of SFA to *n*-9 MUFA (*34*). The fact that SCD did not affect the CLA concentration amid decreases in other biosynthesized FA suggests a preference of this enzyme for palmitoleic and stearic acid as substrate rather than VA as reported by Paton and Ntambi (*35*). Likewise, Herdman et al. (*17*) related their observed tendencies with the presence of different observed isoforms for SCD.

Lipid Peroxidation and Antioxidant Status. The results from TBARS investigations showed that the muscle of treatment animals was resistant to lipid peroxidation for up to 15 min of reaction time, because there were no differences between groups at the start point and only a slight tendency of elevation in treatment animals at the 15 min time point. Lipid peroxidation was 28% higher in treatment animals after 120 min (**Figure 1**). In a previous study (*6*), the differences in muscle lipid peroxidation between groups were reported to occur much more quickly than found in the present study; significant differences were observed after 15 min. Furthermore, the differences between groups were higher (52%) after 120 min of reaction time. Likewise, Mercier et al. (*36*) found highly significant differences in muscle lipid peroxidation between grass-fed animals and mixed diet-fed animals at different reaction times even before chemical oxidation (at a 0 h start point), but, contrary to our results, they found lower lipid peroxidation in grass-fed animals. The concentration of *n*-3 PUFA in muscle of treatment animals was as high as 56 mg/100 g of fresh muscle; it seems that these animals have an appropriate potential to balance lipid peroxidation at a low but not a high accumulation of reactive substances at the times evaluated. This suggests that the increase in some endogenous enzyme activities and the significantly higher concentration of β-carotene found in the present study could act to balance lipid peroxidation only under low oxidative conditions.

For endogenous enzyme activities in beef muscle, the effect of diet is controversial. Some studies have found a clear significant effect on CAT, SOD, and GSH-Px (*16*), whereas others have found effects on only some of these parameters (*9*, *15*, *20*) or no effects at all (*7*). In the present study, only the activity of CAT was significantly higher in treatment animals compared to control animals, whereas no significant changes were observed in GSH-Px and SOD activities (**Table 3**). For GSH-Px, similar to the present study, Descalzo et al. (*9*) found slightly higher, but not statistically significant, activity in muscle of grass-fed animals. The absence of any quantitative appreciable differences of diets on GSH-Px activity has been attributed to high variation among groups (*9*, *37*). The different effects of diet on each endogenous antioxidant enzyme could be related to unique mechanisms of action and the specific conditions generated by diets in each study. SOD is a potent protective enzyme that can selectively scavenge the superoxide radical ($O_2^{\bullet -}$) by catalyzing its dismutation to H_2O_2 and molecular oxygen (O_2). The other antioxidative enzymes, CAT and GSH-Px, act to decompose H_2O_2 to water. According to Mete et al. (*38*), CAT protects cells against high H_2O_2 levels, whereas a GSH-Px-dependent mechanism is more sensitive to low concentrations. In bovines, Mercier et al. (*36*) reported no correlation between CAT and TBARS, and no data were given for GSH-Px. In general, there is a lack of information about correlations between oxidative conditions and antioxidant enzyme activities in bovines. In lambs, Petron et al. (*39*) studied the influence of different pastures on the antioxidant status and oxidative stability of meat and found a significant positive correlation between CAT activity and lipid peroxidation measured as TBARS ($r = 0.58$; $p < 0.01$) and between GSH-Px activity and protein oxidation measured as free thiol content (0.44; $p < 0.05$).

In reference to β-carotene, its high concentration in the treatment diet and muscle could affect lipid peroxidation in different ways. The concentration of β-carotene in longissimus muscle from beef cattle fed on pasture ranges between 0.16 and 0.74 mg/kg of muscle, which is higher (up to 10-fold) than in muscle of grain-fed cattle (*37*). In the present study, the concentration of β-carotene in muscle of treatment animals fed grass silage enriched with *n*-3 PUFA was much higher, up to 2 mg/kg of fresh muscle (**Table 4**), and exhibited a significant positive correlation to lipid peroxidation. Possibly, β-carotene may function as an antioxidant under low accumulation of reactive substances, but its role may change to pro-oxidant, or it may be degraded, under higher accumulation. Łukaszewicz et al. (*40*) studied the susceptibility of lipids from different flax cultivars to peroxidation and its repression by added antioxidants. They found that higher β-carotene concentrations gave rise to increased TBARS levels, conceivably as the result of a pro-oxidative action of high amounts of β-carotene or its degradation to TBARS. Nevertheless, little information is available about the effect of a high concentration of β-carotene in muscle on oxidative stability (*41*).

In general, carotenoids and β-carotene seem to be health promoting, but they may take on circumstantially adverse properties when given in high doses and in the presence of highly oxidative conditions. They may further undergo decay to generate nonradical products and may terminate radical reactions by binding to attacking free radicals (*42*). On the other hand, the tendency for a lower concentration of α-tocopherol in muscle of treatment animals could affect the antioxidant balance power at higher accumulation of reactive substances, and despite higher α-tocopherol incorporation into tissue, lower TBARS were measured (significant negative correlation). Among all antioxidants present in beef, α-tocopherol is believed to play a key role in oxidative stability and extent of lipid peroxidation (*41*). Hence, the low concentration of α-tocopherol, as well as a similar amount of retinol and SOD activity, in treatment animals prevented the higher levels of β-carotene and CAT activity from being reflected in a higher AOC measured by both assays in both extracts. Thus, no diet effect was found on AOC of muscle of German Holstein bulls (**Table 5**). Mahecha et al. (*20*) also found an effect of diet on individual antioxidants; however, the antioxidative status of muscle from German Simmental bulls as measured by TEAC and FRAP assays was not significantly affected. Only Descalzo et al. (*9*) have reported a significant effect of diet on the ferric reducing potential of muscle measured by the FRAP assay, but no effect was seen on radical scavenging capacity measured by the ABTS assay, which functions similarly to the TEAC assay. A significant association between higher concentrations of almost all measured antioxidants (α-tocopherol, β-carotene, ascorbic acid, glutathione content, and SOD activity) with a high, but not statistically significant, increase in GSH-Px activity was found to exist in grass-fed animals compared to grain-fed animals. Similar to Descalzo et al. (*9*), Gatellier et al. (*16*) also reported an increased effect of diet on AOC measured by the FRAP assay. Such is not the case in the present study, where both assays failed to show an effect of diet with increasing reaction time. On the other hand, results from the present study and previous results revealed the need to measure both assays at a minimum time point of 30 min because AOC after 5 min (normal time used for these assays) was only ≤50% of the final value obtained at 60 min, whereas after 30 min, it was between 72 and 83%.

Color Stability. The color considerably influences beef's acceptability and purchasing decisions at retail points (*15*). Among the various measured parameters of beef color, redness is considered to be the most important because changes in color from red to brown (decrease of *a**) indicate oxidation of oxymyoglobin to metmyoglobin (*16*). In the present study, no differences were found in the redness of beef from animals fed either diet. Gatellier et al. (*41*) found that the antioxidant α-tocopherol delays myoglobin oxidation and extends color stability on retail beef. Likewise, Yin and Cheng (*43*) reported that in an "oxymyoglobin: liposome model" the presence of β-carotene (at levels similar to those found in pasture-fed steers) delayed myoglobin oxidation. Although a strong relationship has been reported between lipid peroxidation and myoglobin oxidation (*44*), it is unclear if protein and lipid oxidation are concomitant processes or if one event precedes the other. In beef, Arnold et al. (*45*) demonstrated that lipid peroxidation and metmyoglobin formation occurred simultaneously but with different progression patterns at α-tocopherol levels of < 3 μg/g of muscle. However, no description has been reported that takes into consideration both α-tocopherol and β-carotene levels. Insani et al. (*15*) reported that dietary differences among cattle had a minor impact on protein oxidation in fresh meat but that differences could be appreciable by aging or storage. On the other hand, the slight reduction of lightness in treatment animals could also support the hypothesis of low oxidative conditions. The literature generally reports that pasture- or forage-finished cattle had darker colored beef muscle (lower *L**) when compared with grain-fed cattle (*25*, *46*). This has been attributed to differences in ultimate pH values, sex, and grade of oxygenation (*46*), age at slaughter, higher activity, and fat content (*12*). In the present study there were no differences between groups for pH (5.6 vs 5.6), sex, or change in lightness that could be attributed first to the lower fat content (sum SFA, MUFA, and PUFA) in muscle of treatment animals (2367 vs 1765 mg/100 g of muscle, control and treatment animals respectively, **Table 2**) or possibly to a small reduction in oxygen due to low oxidative conditions in treatment animals.

ABBREVIATIONS USED

SFA, saturated fatty acids; LDL, low-density lipoprotein; CHD, coronary heart disease; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; FA, fatty acids; SCD, stearoyl-CoA-desaturase; Δ6d, Δ6-desaturase; Δ5d, Δ5-desaturase; AOC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; v, volume; GC, capillary gas chromatography; MDA, malondialdehyde; FRAP, ferric reducing antioxidant power; TEAC, Trolox equivalent antioxidant capacity; TPTZ, 2,4,4-tri(2-pyridyl)-*s*-triazine; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid; $K_2S_2O_8$, potassium peroxodisulfate; PBS buffer, phosphate-buffered saline; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; CAT, Ccatalase; SOD, superoxide dismutase; pyrogallol, 1,2,3-trihydroxybenzene; DTPA, diethylenetriaminepentaacetic acid; GSH-Px, glutathione peroxidase; EDTA, ethylenediaminetetraacetic acid; BHT, butylhydroxytoluene; HPLC; high-performance liquid chromatography; GLM, general linear model; LMS, least-squares mean; SEM, standard error of LSM; CLA, conjugated linoleic acid; VA, vaccenic acid.

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