CUMULATIVE THESIS

Aus dem Leibniz-Institut für Nutztierbiologie in Dummerstorf

Diätetische Effekte auf die Beziehung zwischen dem Fettsäureprofil, der Lipidperoxidation, dem antioxidativen Status und der Qualität in Rindermuskel

Dissertation

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Erlangung des akademischen Grad

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Dietary effects on the relationship between fatty acid profile, lipid peroxidation, antioxidant status, and quality in beef muscle

Thesis

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Dedicatory

To God: *for having permitted to come up to this point and to have given me health to achieve my goals.*

To my dear husband, best friend, and colleague, Joaquin: *this thesis is dedicated to you in a very special way for your practical and emotional support. Without your unconditional support in this trip that we tackled together, I doubt that my doctoral studies have been completed.*

To my children's, Santiago and Gabriela: *you have been great sources of motivation and inspiration.*

To my parents: *who taught me that the best richness for the man is the knowledge, and the best kind of knowledge to have is that which is learned for its own sake.*

To my siblings: *for their support and example during all my life*

To my nephew and nieces: *to whom I expect to show the importance of studying for the personal and professional development*

To my family in law: *for helping me when more I have needed it*

Quotes

Always remember that
for those who persist,
today's dreams are transformed
into tomorrow's successes.

~ Kelly D. Caron

Believe in the impossible,
hold tight to the incredible,
and live each day to its fullest potential.
You can make a difference
in your world.

~ Rebecca Barlow Jordan

Abbreviations

AA	Arachidonic acid
ABTS	2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid
ACC	Acetyl-CoA Carboxylase
AOA	Antioxidant activity
AOC	Total antioxidant capacity
BHT	Butylhydroxytoluene
CAT	Catalase
CLA	Conjugated linoleic acid
CHD	Coronary heart disease
CHO-rich	Carbohydrate rich
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
Δ6d	Δ6-desaturase
Δ5d	Δ5-desaturase
DTPA	Diethylene-triamine-pentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAS	Fatty-acid synthase
FRAP	Ferric Reducing Antioxidant Power
GC	Capillary gas chromatography
GLM	General linear model
GSH-Px	Glutathione Peroxidase
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein

HPLC	High-performance liquid chromatography
IMF	Intramuscular fat
$K_2S_2O_8$	Potassium peroxodisulfate
LDL	Low density lipoprotein
LSM	Least square mean
MEI	Metabolisable energy intake
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acids
ORAC	Oxygen Radical Absorbance Capacity
PBS-buffer	Phosphate buffered saline
Pyrogallol	1,2,3-Trihydroxybenzene
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SCD	Stearoyl-CoA-desaturase
SEM	Standard error of LSM
SFA	Saturated fatty acids
SOD	Superoxide dismutase
SumFA	Total sum of fatty acids
TCA	Trichloric acid
TG	Triglyceride
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox Equivalent Antioxidant Capacity
TR	Thioredoxin Reductase
TPTZ	2,4,4-Tri(2-pyridyl)-s-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
V	Volume
VA	Vaccenic acid

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Chapter 1

General Introduction

1.1. Introduction

It has been demonstrated that quality and total quantity of fatty acids (FA) intake plays an essential role in determining human health problems (Ramsden et al. 2009). Fat quality of foods has been related with the presence of FA influencing the human health maintenance and disease prevention (Uauy, 2009). It has stimulated the consumer's interest on the fatty acid composition of different animal fat depots and products, and has a larger effect for some consumer choices than price. It has also raised the investigations to improve the fatty acid composition of meat and products (Lusk & Parker, 2009). In beef, intramuscular fat depot (IMF) is of most interest in relation to fatty acid composition and human health. The IMF of different European bovine breeds consists on average of 43-48 % saturated FA (SFA), 37-48 % monounsaturated FA (MUFA, one double bond), and 3-9% polyunsaturated FA (PUFA, more than 1 double bond) of total FA, respectively (Nuernberg et al. 2009; Ratnayake & Galli, 2009). Palmitic (C16:0) and stearic acid (C18:0) are the major SFA in beef. There is very strong evidence that myristic (C14:0) and palmitic acid raises serum cholesterol levels and this occurs predominantly by increasing bad cholesterol (Low density lipoprotein, LDL) levels (Kris-Etherton & Yu, 1997). These FA account for most of the cholesterol-raising activity from beef, thereby increasing the risk of atherosclerosis, cardiovascular disease and stroke. Instead, several studies have shown that C18:0 has a neutral effect on total cholesterol; then, its effect is minimal and not detrimental to human health. Beef is also a source of two SFA, lauric (C12:0) and C14:0, which are related to a number of bad effects on human health issues. Although there is small amount of these FA in beef (less than 1% and 2-3%, respectively), they are responsible for raising bad cholesterol levels in blood serum and have been shown to be strongly correlated with early heart attack (Abbas et al. 2009). Contrary to SFA, MUFA, and PUFA are generally regarded as beneficial for human health (Abbas et al. 2009). The primary MUFA in beef is oleic acid. A number of epidemiological studies comparing disease rates in different countries have suggested an inverse association between MUFA intake and mortality rates to

cardiovascular disease (CVD) (Daley et al. 2010). Likewise, Paniagua et al. (2007) found that weight maintenance with a MUFA-rich diet improves insulin sensitivity, as indicated by lower homeostasis model analysis-insulin resistance compared with carbohydrate rich (CHO-rich) and high-SAT diets. Ingestion of a virgin olive oil-based breakfast (rich in oleic acid) decreased postprandial glucose and insulin concentrations, and increased HDL (High density lipoprotein) concentrations as compared with CHO-rich diet. Other MUFA that has been related to benefits for human nutrition is *trans*-11 vaccenic acid (VA), the predominant *trans* C18:1 isomer in ruminant fat and the major precursor to the endogenous synthesis of *cis*-9,*trans*-11 conjugated linoleic acid (CLA) in humans and animals (Griinari & Bauman, 1999). Bassett et al. (2010) found that vaccenic acid-rich butter protects against atherosclerosis in LDL receptor deficient LDLr–/– Mice. Results from Miller et al. (2003) showed that VA is converted to *cis*-9,*trans*-11 CLA in human mammary (MCF-7) and colon (SW480) cancer cell lines and VA influenced cell viability and other CLA bio-responsive markers. However, the effect of VA in cancer is potential, but contradictory and more research is necessary in this area since some epidemiological studies in human indicate that the intake (by estimating diet or measuring serum lipids) of VA may increase the risk of cancer (Field et al. 2009). The same happens with other monounsaturated *trans* FA which negative effects on lipoproteins and heart disease are unclear. According to Brouwer et al. (2010), although most of studies show that these FA raise the ratio of plasma LDL to HDL cholesterol (Mozaffarian et al. 2006; Almendingen et al. 1995), some epidemiological studies have showed no association between ruminant *trans* fatty acid intake and heart disease risk (Jakobsen et al. 2008; Pietinen et al. 1997). Data of the effects on plasma lipoproteins in humans are limited. In reference to PUFA, CLA (main isomer *cis*-9,*trans*-11 CLA) have mainly captured the attention in human nutrition. *Cis*-9,*trans*-11 CLA has been shown to have anticancerogenic properties in various animal studies (Kelly et al. 2007). Different human cancer studies have found an inverse association between the level of *cis*-9,*trans*-11 CLA in the diet and the risk of developing cancer in breast adipose tissue. The mechanisms whereby this occurs are not known yet, but

some theories are that *cis*-9,*trans*-11 CLA reduces cell proliferation, alters various components of the cell cycle, and induces apoptosis (Dhiman et al. 2005). *Trans*-10,*cis*-12 CLA is another linoleic acid isomer that has been shown to have biological activity and has been promoted with human benefits (Brouwer et al. 2010). This CLA isomer has been shown to reduce obesity in animals and triglyceride (TG) accumulation in adipocyte cell culture models. This has led to its promotion as a weight-loss supplement in humans (Miller et al. 2008). Additionally to CLA, there are two important families of PUFA – *n*-3 and *n*-6 FA. The major FA found in beef are *α*-linolenic acid (C18:3*n*-3), eicosapentaenoic acid (EPA) (C20:5*n*-3), and docosahexaenoic acid (DHA) (C22:6*n*-3) from *n*-3, and linoleic acid (C18:2*n*-6) and arachidonic acid (C20:4*n*-6) from *n*-6 (Abbas et al. 2009). Both fatty acid families are important constituents of all cell membranes and essential for survival of humans and other mammals (Chang et al. 2009). Because *n*-6 FA are the precursors of pro-inflammatory eicosanoids, higher intakes have been suggested to be detrimental (Willet, 2007) while a higher intake of *n*-3 PUFA has been associated for reducing cardiovascular problems and mortality and the ratio of *n*-6 to *n*-3 FA has been suggested 4/1 for human health (Simopoulos, 2008). However, there is a great controversy about it. Willet (2007) considers that this hypothesis is based on minimal evidence, and in humans higher intakes of *n*-6 FA have not been associated with elevated levels of inflammatory markers. Then, this author mentions that adequate intakes of both *n*-6 and *n*-3 FA are essential for preventing cardiovascular disease and type 2 diabetes, but the ratio of these FA is no useful. Ramsden et al. (2009) and Deckelbaum & Calder (2010) mention that both families interact with one another in a complex network and their accumulation and activity depend on both their relative and absolute intake, the intake of both is considered important in the context of one another. Despite this controversy, the increase in the consumption of *n*-3 FA continues being considered more beneficial for human health (EFSA, 2010), and continues impacting demand by consumers Meat and dairy products derived from ruminants are sources of *n*-3 PUFA and the main dietary sources of CLA and VA (Dhiman et al. 2005; Field et al. 2009). Therefore, their accumulation in

ruminant's products is an important marketing strategy for beef and a main topic in current research of farm animals.

It has been demonstrated that the primary way to increase beneficial FA in beef is the diet (Scollan et al. 2006). The use of diets based on forage and/or the supplementation of vegetable oils have been found positive to increase the sum of *n*-3 PUFA in beef with different results on individual *n*-3 PUFA, CLA and VA. Feeding fresh grass or grass silage compared to concentrates, rich in C18:3*n*-3 and C18:2*n*-6, respectively, resulted in higher percentage of *n*-3 PUFA and *cis*-9,*trans*-11 CLA, but not in higher concentration (mg/100 g of sample) of CLA in muscle lipids (Nuernberg et al. 2005). Fish oil-supplemented diets increased the percentage of *n*-3 FA, *cis*-9,*trans*-11 CLA, and VA in *longissimus* muscle of steers (Wistuba et al. 2007). Beef bulls fed concentrate supplemented with fish oil in different amounts increased muscle concentrations of the *n*-3 PUFA, EPA, and DHA as well as VA compared with control; however, there was no effect of treatment on the concentration of *cis*-9,*trans*-11 CLA (Waters et al. 2009). Four groups of bulls were assigned to different diets, mainly differing in the presence of linseed as the predominant *n*-3 fatty acid source in the concentrate either or not in combination with grass (silage) as the roughage. The results show that feeding *n*-3 PUFA during the phases before the finishing diet increased the long chain *n*-3 PUFA (C20:5*n*-3, C22:5*n*-3, and C22:6*n*-3, mg/100 g sample) compared to animals which were fed only a concentrate enriched with C18:3*n*-3 in the finishing period. The *cis*-9,*trans*-11 CLA content was increased by feeding linseed in the fattening period (Raes et al. 2003). The effects of the type of grass silage and dietary inclusion of FO on the fatty acid profile of bovine intramuscular fat were investigated by Noci et al. (2007). Wilting of grass prior to ensiling increased the concentration of *cis*-9,*trans*-11 CLA, but did not affect the *n*-6/*n*-3 PUFA ratio. Increasing FO supply linearly increased the concentration of the *cis*-9,*trans*-11 CLA and VA and linearly decreased the *n*-6/*n*-3 PUFA ratio. Research continues to find a significant increase of all beneficial FA without detrimental effect on beef quality.

The effect of PUFA enriched diets on the fatty acid composition of bovine muscle has been evaluated under unrestricted conditions, but to our knowledge not under feeding restriction. However, results obtained in cows fed the same diet with different levels of energy (Cabaraux et al. 2004), and results from other species (Hynes et al., 2003) allowed to hypothesize that the fatty acid composition of bovine muscle can be affected by a combined effect of dietary fatty acid composition and energy restriction due to metabolic adaptation to energy restriction and/or due to alterations in rumen environment. It is considered that a negative energy balance leads to a unique scenario in which certain FA are preferentially retained while others are oxidized (Chen & Cunnane 1992, Cha & Jones, 1996). It appears that adipose tissue concentrations of linoleic acid (18:2*n*-6) increases under situations of energy restriction whereas shorter chain FA are favored for oxidation as demonstrated in growing rats (Cha & Jones 1996) and cows (Cabaraux et al. 2004). As well, short-term food restriction in rats increased concentrations of C18:0, C18:2*n*-6, C20:4*n*-6, and C22:6*n*-3 whereas caused reductions in C16:0, C16:1, and C18:1*cis n*-9 in serum triglycerides and hepatic tissues of these animals (Chen & Cunnane 1992). On the other hand, feeding restriction of reindeers caused alteration of the rumen environment (Nilsson et al. 2006). Feeding restriction is a normal practice use by farmers in different conditions. It is used like strategy for managing heat load in feedlot cattle. Problems in managing cattle exposed to elevated climatic temperatures may be further complicated if cattle are being fed high-energy diets; then, reducing metabolisable energy intake (MEI) through feeding restriction could allow a lower heat production and enhance feed conversion (Mader et al. 2002). It also is used in some countries that depend largely on grazing to support animal production where the natural conditions sometimes reduce drastically the food availability (Abegaz et al. 1996). Additionally, some farmers like to use it like a way of increasing growth rate in some periods by the use of the compensatory growth phenomenon. It is known as the ability of an animal to exhibit, after feeding restriction, greater growth rates than in unaffected animals of the same chronological age (Wilson & Osbourn, 1960). Moreover, this management has increased the attention on

beef tenderness without clear results yet. Therkildsen et al. (2008) did not find effect of feeding restriction on *longissimus* muscle, but on semitendinosus muscle tenderness from Friesian bull calves while Purchas et al. (2002) found higher tenderness in *longissimus* muscle from unrestricted bulls than restricted when they got different age at slaughter, but not when they got the same age. Likewise, restricted bulls produced more tender muscle after a compensatory growth (Hornick et al. 1998).

Additionally, interest is given to the conservation of the beneficial FA for human consumption after the slaughter of animals due to the susceptibility of unsaturated FA to destruction by reactive oxygen species (ROS) (Santé-Lhoutellier et al. 2008; Chaijan, 2008). ROS may damage biomolecules directly, or initiate chain reactions in which ROS are passed from one molecule to another. This can result in extensive damage not only of lipids, but also of other cell structures such as proteins (Fyfe, 2000). In the case of lipids, the process is called lipid peroxidation (Chaijan, 2008). The exposure of living organisms to ROS is unavoidable in aerobic life since the generation of ATP from molecular oxygen demands electrons. ROS fall into two groups; those that contain unpaired electrons (O_2 -, OH-), or those that have the ability to extract electrons from other molecules (H_2O_2, HOCl) (Fyfe, 2000). Lipid peroxidation in muscle foods is initiated by several components arising from both internal and external sources (Terevinto et al. 2010) (Figure 1.1). In beef, lipid peroxidation is accentuated in the immediate post-slaughter period, during handling, processing, storage, and cooking. The lipid peroxidation leads to discoloration, drip losses, off-odor, and off-flavor development, texture defects and the production of potentially toxic compounds. For these reasons, it is one of the main factors limiting the quality and acceptability of meats and other muscle foods (Chaijan, 2008). The most important aspect increasing lipid peroxidation in beef is the imbalance between antioxidant defense and the oxidative process (Descalzo et al. 2007; Mercier et al. 2004; Gatellier et al. 2005). The muscle antioxidant defense is composed by non-enzymatic hydrophilic and lipophilic compounds like vitamin E, vitamin C, carotenoids, ubiquinols, polyphenols,

cellular thiols, and enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-px) (Descalzo et al. 2007) (Figure 1.1). The concentration of the antioxidant components can be measured individually and the knowledge of their contents can be useful to understand the relationship between antioxidant status and lipid peroxidation. However, these measurements may be time- and cost-consuming not all components which show antioxidant properties in the tissue will be considered. In addition, the combined effects among the antioxidants, and any compensation mechanism due to relative deficiency in any one antioxidant are some arguments to consider that the total antioxidant capacity (AOC), known as "cumulative action of all the antioxidants present in plasma and body fluids" (Ghiselli et al. 2000). The measurement by one specific method may give more biologically relevant information than that obtained from measuring concentrations of individual antioxidants (Ghiselli et al. 2000; Thorat et al. 2009; Suresh et al. 2009). The measurement of AOC is a matter of growing interest because it may provide a variety of information, such as resistance to oxidation or quantitative contribution of antioxidant substances (Serrano et al. 2007). According to Ghiselli et al. (2000) the terms AOC and antioxidant activity (AOA) are often used interchangeably, but they are quite distinct. AOA corresponds to the rate constant of a single antioxidant against a given free radical. AOC is the measurement of the content of a given free radical scavenged by a test solution, independently from the antioxidant activity of any one antioxidant present in the mixture. For Descalzo et al. (2008), AOA is the capability of a compound (composition) to inhibit oxidative degradation, e.g. lipid peroxidation while AOC gives the information about the duration of anti-oxidative reaction, the reactivity characterizes the starting dynamics of reduction at a certain concentration of an antioxidant or antioxidant mixture. In our understanding, AOC will be used to refer to a full spectrum of antioxidant activity against reactive radicals in a special time point and in this way to give a general idea of the quantitative contribution of antioxidant substances to the antioxidant defense in beef under the evaluated conditions. Two different assays will be used to access to this information. It has been demonstrated that only one

method should not be used to assess AOC in beef (Wu et al. 2008). Beef is a complex matrix and a single method cannot cover all chemical structures which show anti-oxidative properties in beef tissue. This was also proposed for other biological systems (Prior & Cao, 1999). Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Antioxidant Power (FRAP) assays are commonly used methods for assessing AOC. TEAC assay measures the ferric to ferrous reduction in presence of antioxidants and is based on the principle of inhibition of radical caption production by antioxidants in the sample. The concentration of antioxidants in the sample is inversely proportional to the absorbance of radical cation produced by 2,2´-azino-bis-(3-ethyl benzothiozoline -6 sulfonate) (ABTS) (Gupta et al. 2009). FRAP assay measures a ferric to ferrous ion reduction at low pH which causes a colored ferrous-tripyridyltriazine complex. FRAP assay compares the absorbance in test reaction mixtures with those containing ferrous ions in known concentration to determine antioxidant levels (Benzie & Strain, 1996). Different factors can affect the susceptibility of beef to lipid peroxidation (Tres et al. 2008), the antioxidant status, and the balance between them. However, there is a dearth of investigations regarding to feeding restriction. Because of these reasons it is very important to have knowledge about the balance between antioxidant status and lipid peroxidation in basal diets used for bovines and especially in enriched PUFA diets under different conditions of management. In this way, it would be possible to give recommendations under different diets and conditions for a real increase of beneficial FA in beef with a guarantee of quality to the consumers.

To our knowledge: i) it is the first research in bulls that give information together about endogenous and exogenous antioxidants in beef muscle (tocopherols, carotene, endogenous enzymes, and trace elements), their relationship with AOC and lipid peroxidation comparing diets enriched with *n*-6 and *n*-3 FA without using additional supplementation of antioxidants. Some studies published until now, however have been carried out with basal diets and/or supplemented with *α*-tocopherol. ii) It is also the first time that this information is given in feeding restricted animals under the mentioned

conditions. iii) It is the first research which gives information about total AOC of fresh and stored beef muscle using FRAP and TEAC assays with special emphasis to both hydrophilic and lipophilic active antioxidants at different reaction times.

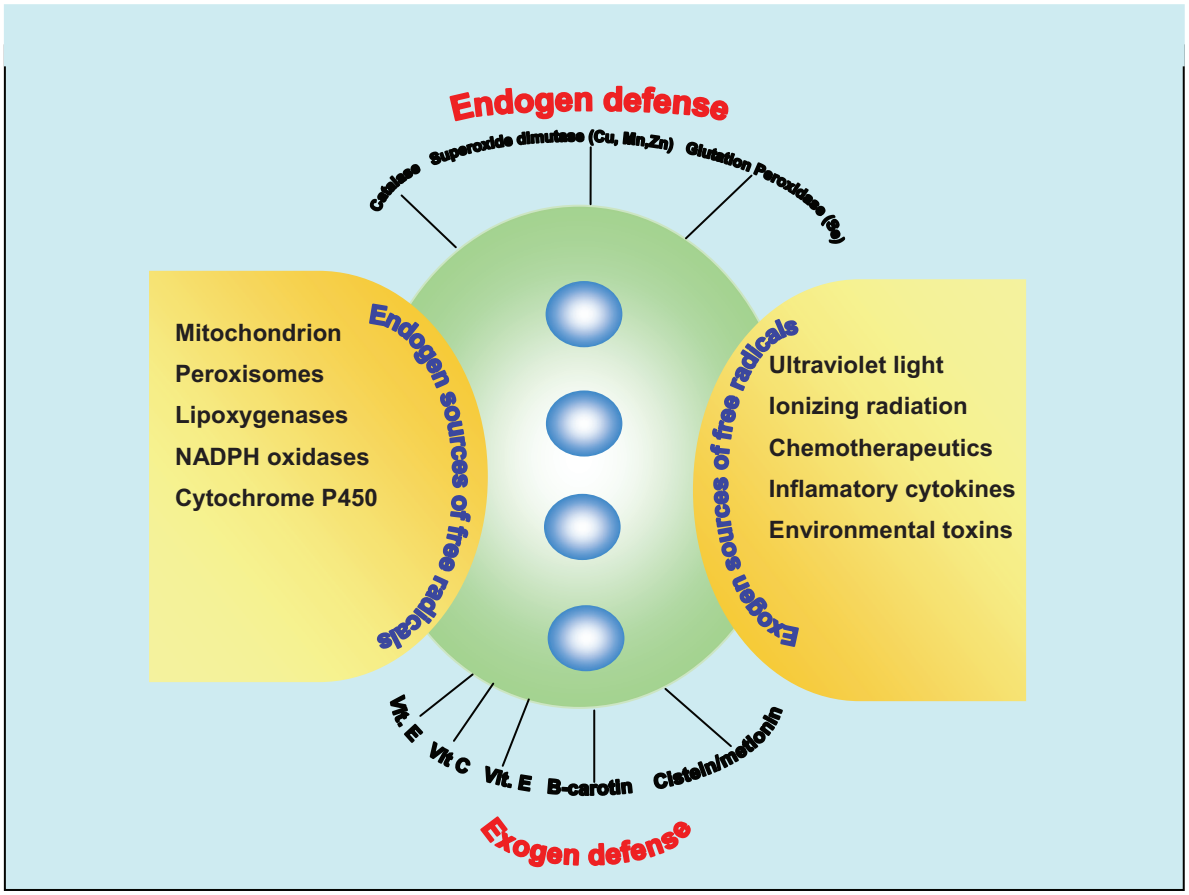

Figure 1.1 Oxidative stress and Antioxidant capacity

Source: Adapted from Wien (2001)

1.2. Objectives and structure of the thesis

Two different experiments were carried out in the frame of the investigation. The **first** one with Simmental German bulls under indoor conditions comparing *n*-6 and *n*-3 PUFA enriched diets. *N-6* enriched diets were considered as control and *n*-3 enriched diets as treatment diet in both experiments. In the experiment 1, *n*-6/*n*-3 ratio was decreased twofold in the treatment diet. This was given by a light increase of *n*-3 PUFA. The control group (*n* = 9) were daily fed maize silage/grass silage (70/30, *ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including soybean (2 kg the first 112 days, 2.5 kg the next 110 days, and 3 kg the rest of the time). Treatment group I, consisted of unrestricted animals (*n* = 7), fed grass silage (*ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including rapeseed in the same proportion as the control group. Treatment group II, consisted of restricted animals (*n* = 9), fed as treatment group I with a restriction of 1 kg of concentrate (50%) per day during the first 112 days of the fattening period (approximately 10 MJ/d of metabolizable energy intake, MEI). This experiment was done using a single fixed kept feeding system.

In the **second one**, *n*-6/*n*-3 ratio was decreased also to the half in the treatment diet. This decrease was given by a strong increase of *n*-3 PUFA. Control group consisted of animals (*n*=15) fed maize silage, and concentrate enriched with *n*-6 FA (soybean-based concentrate); and treatment group consisted of animals (*n*=14) fed grass silage, and concentrate enriched with *n*-3 FA (linseed oil and rapeseed cake based concentrate). This experiment was done using a group keeping feeding system. Bulls from both experiments were slaughtered by captive bolt stunning followed by exsanguinations in the abattoir of the Leibniz Institute for Farm Animal Biology in Dummerstorf (Germany). The slaughter and dressing procedures were in accordance with EU specifications.

The main objective of this research was to investigate the influence of feeding *n*-3 and *n*-6 PUFA enriched diets on the relationship of lipid

peroxidation and antioxidant status including fatty acid composition and quality in *longissimus* muscle from bulls.

To approach the overall research objective, five detailed objectives were selected:

To evaluate the **effect of diet** on the relationship between lipid peroxidation, some individual antioxidants, fatty acid profile, and the quality in *longissimus* muscle of German Simmental bulls comparing control animals fed concentrate including soybean and a mixture of maize silage and grass silage (70:30) (*n*-6 enriched diet) *versus* restricted and unrestricted animals fed concentrate including rapeseed and only grass silage (*n*-3 enriched diet) (Chapter 2).

To investigate **the effect of storage** (14 days of storage *versus* 24 h post-mortem) **on fatty acid composition and fat soluble vitamins** in *longissimus* muscle of German Simmental bulls fed an *n*-3 PUFA enriched diet or an *n*-6 PUFA enriched diet (Chapter 2).

To evaluate **the effect of diet on the antioxidant enzyme activities** in *longissimus* muscle of German Simmental bulls comparing control animals fed concentrate including soybean and a mixture of maize silage and grass silage (70:30) (*n*-6 enriched diet) *versus* restricted and unrestricted animals fed concentrate including rapeseed and only grass silage (*n*-3 enriched diet) (Chapter 3).

To evaluate FRAP and TEAC assays to measure **the effect of diet and storage on antioxidant capacity in** *longissimus* muscle of German Simmental bulls fed an *n*-3 PUFA or *n*-6 PUFA enriched diet using different reaction times with special emphasis to both hydrophilic and lipophilic active antioxidants at different reaction times (Chapter 3).

To evaluate **the relationship between lipid peroxidation and antioxidant status, the fatty acid profile and the color stability** of *longissimus* muscle in

German Holstein bulls comparing control animals fed concentrate including soybean and maize silage (*n*-6 enriched diet) *versus* animals fed concentrate including rapeseed cake and linseed oil, and grass silage (*n*-3 enriched diet) (Chapter 4).

Chapter 2

Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls

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Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls

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ABSTRACT

This study evaluated the effect of diet on fatty acid profile, vitamins, trace elements, lipid peroxidation, and quality of *longissimus* muscle of German Simmental bulls. The effect of storage on fatty acid profile and vitamins was also included. A control group was fed concentrate including soybean, and maize silage/grass silage. Treatment group I (unrestricted) was fed concentrate including rapeseed, and grass silage. Treatment group II (restricted) was fed like treatment group I with a feed restriction period. The treatment diet was not effective to give similar daily live weight gain to the control diet, but it was successful in improving beef fatty acid composition without affecting tenderness and color (under unrestricted conditions). There were no differences in vitamins and cooking loss, but selenium decreased in treatment groups. Stimulated lipid peroxidation, in samples taken immediately post-mortem, was higher in treatment groups. Polyunsaturated fatty acids decreased, saturated fatty acids and intramuscular fat increased after 14 days of storage while vitamins had no significant reduction.

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Key words: Beef, *n*-3 PUFA, Grass silage, Maize silage, Vitamins, TBARS, Storage

1. Introduction

A high incidence of mortality in Europe resulting from cardiovascular problems associated with the actual diet has been recently found by Muller-Nordhorn, Binting, Roll, & Willich (2008). Studies like this have increased the interest of consumers in healthy food. For beef, much attention has been given to lipids, nutrition being the major route for increasing the content of beneficial fatty acids in beef (Scollan et al., 2006). It is well known that part of dietary 18:2*n*-6 and 18:3*n*-3 fatty acids can escape ruminal biohydrogenation and go on to be deposited in the tissues. However, most of them are extensively metabolized and biohydrogenated in the rumen (Harfoot & Hazlewood, 1997). The rumen microbial ecosystem yields not only the final product: stearic acid (18:0), but a range of biohydrogenation intermediates such as monounsaturated *trans*, cis, and conjugated fatty acids (Jouany, Lassalas, Doreau, & Glasser, 2007). Some of these intermediates are transported via duodenal digesta and deposited finally in the tissues (Scollan et al., 2006). Therefore, it is really important that producers, industry, and researchers generate alternatives to improve beef quality under the actual beef production conditions. A common diet used for beef cattle production under indoor conditions in Europe consists of maize silage:grass silage with a higher proportion of maize silage, and a concentrate including soybean. A high inclusion of maize silage in beef cattle diets has been related to a high amount of linoleic acid (18:2*n*-6), high *n*-6/*n*-3 ratio, and low vitamin E contents in beef (O'Sullivan et al., 2002). In the same way, the use of grain and soybean has also been related with an increase of *n*-6 PUFA and an unbalance in the *n*-6/*n*-3 ratio (Kim, Adesogan, Badinga, & Staples, 2007) which is not desired for human health. Hence, the first purpose of this study was to evaluate an alternative diet in order to increase beneficial fatty acids in beef, without affecting other parameters of beef quality. Likewise, it was considered that just as the improvement of beef quality using different

feeding strategies is important for actual and future beef production systems, the maintenance of quality after slaughter is necessary in order to guarantee quality to the consumers. This study had two objectives: (i) to evaluate fatty acid profile, trace elements, fat soluble vitamins, lipid peroxidation, and quality of *longissimus* muscle of German Simmental bulls comparing control animals fed concentrate including soybean and a mixture of maize silage and grass silage (70:30), versus restricted and unrestricted animals fed concentrate, including rapeseed and only grass silage, (ii) to investigate the effects of storage (14 days of storage versus 24 h post-mortem) on fatty acid profile and fat soluble vitamins.

2. Materials and methods

2.1. Animals and experimental design

25 male German Simmental calves (3–4 months) were included in an indoor experiment. They were randomly assigned into three groups with different feeding regimes. The control group (n = 9) were daily fed maize silage/grass silage (70/30, *ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including soybean (2 kg the first 112 days, 2.5 kg the next 110 days, and 3 kg the rest of the time). Treatment group I, consisted of unrestricted animals (*n* = 7), fed grass silage (*ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including rapeseed in the same proportion as the control group. Treatment group II, consisted of restricted animals (*n* = 9), fed as treatment group I with a restriction of 1 kg of concentrate (50%) per day during the first 112 days of the fattening period (approximately 10 MJ/d of MEI). All groups included 5% of commercial mineral mix in the concentrate. Vitamins were not supplemented. Details of feeding have been compiled in Table 2.1. All bulls were slaughtered at approximately 635 kg live weight by captive bolt stunning followed by exsanguinations in the abattoir of the Research Institute for the Biology of Farm Animals in Dummerstorf (Germany). The slaughter and

dressing procedures were in accordance with EU specifications. *Longissimus* muscles were taken immediately after slaughter for the thiobarbituric acid (TBARS) assay, and after 24 h chilling for meat quality assessment, fatty acid composition, vitamins, trace elements, and cooking loss. Samples were taken at the 6th–13th rib of the right carcass side. One muscle slice (5 cm) was vacuum-packed and stored in the dark at 2 ºC for 14 days for fatty acids and vitamins analysis. After this time, it was kept at -20 ºC until the respective analysis.

2.2. Tenderness, cooking loss and color

The samples for tenderness (2.5 cm) were cooked in a water bath until the internal temperature of each steak was 70 ºC. After cooling, for 90 min at room temperature, five cores (1.2 cm diameter) were taken from the steaks parallel to the muscle fibre orientation. Each core was sheared using a Texture Analyser Winopal (Ahnbeck) with a Warner–Bratzler blade (1.0 mm wide). The Warner–Bratzler peak force was the measurement considered as tenderness. Cooking loss and color were measured according to the methodology described by Nuernberg et al. (2005).

2.3. Fatty acid composition

The intramuscular fat (IMF) of 2 g muscle was extracted with chloroform/methanol (2:1, v/v) according to Folch, Lees, & Stanley (1956) by homogenization (Ultraturrax, 3 $_{x}$ 15 s, 12000 rpm) at room temperature. The fatty acid composition of muscle lipids and feed was determined using the methodology described by Nuernberg et al. (2002).

Table 2.1 Composition of the diets.

	Concentrate control group%	Concentrate treatment groups%	Maize silage	Grass silage
Chemical composition (%)				
Crude protein	18.90	19.60	9.60	20.10
Crude fat	2.80	4.90	3.10	4.10
Crude Ash	8.30	8.60	5.20	14.10
Acid detergent fiber (ADF)	3.20	5.80	18.20	22.30
ME (MJ/kg)	12.30	12.40	11.00	10.10

Fatty acids (% FAME)	***TMR control group***	***TMR treatment groups***
C4:0	0.14	0.19
C16:0	12.06	7.97
C16:1*cis*-9	0.44	1.43
C18:0	3.03	1.56
C18:1*cis*-9	23.07	35.60
C18:2*n*-6	46.58	26.96
C18:3*n*-3	7.25	8.06
PUFA	54.10	35.31
n-3 FA	7.36	8.21
n-6 FA	46.66	27.02
Ratio *n*-6/*n*-3	6.34	3.29
Vitamins (mg/kg)[a]		
Retinol (A)	13.71	14.79
δ –Tocopherol	0.44	n.d.
γ –Tocopherol	1.80	0.73
α –Tocopherol	18.50	22.30
Trace elements (mg/kg)*		
Zn	24.70	25.50
Se	<0.01	<0.01
Fe	231.00	242.00
Cu	4.45	5.35

n.d.: Not detected.
[a] mg/kg fresh material.

2.4. Lipid peroxidation

For evaluating the stability of the beef muscle samples, postmortem, against stimulated lipid peroxidation, the production of thiobarbituric acid reactive substances (TBARS) was used. TBARS's are expressed in terms of malondialdehyde (MDA), a breakdown product formed during lipid peroxidation. To stimulate lipid peroxidation 3 ml of the muscle homogenate were incubated with 0.1 mM ascorbate and 5 μM $FeSO_4$. From this, 0.5 ml were 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 ºC and after 0, 15, 30, 60, and 120 min 0.5 ml each of this incubated homogenate were pipette into the TCA medium as above. These samples were centrifuged at 10,000g for 10 min and 0.5 ml of the supernatants were mixed with 0.5 ml thiobarbituric acid (0.67%) and boiled for 15 min in a water bath. After immediate cooling, the absorbance at 535 nm was determined. Standard MDA solution was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane, and the results were expressed as mg/kg of sample (Kuechenmeister et al., 1999).

2.5. Determination of fat soluble vitamins

Retinol (vitamin A), δ-tocopherol, γ-tocopherol, α-tocopherol, and β-carotene were analyzed in the muscle samples according to the adaptation of the methodology described by Linden (2003) and Ryynanen, Lampi, Salo-Vaananen, Ollilainen, & Piironen (2004). Three sub-samples were prepared by homogenizing tissue (4 g per sub-sample) in 6 ml of a mixture composed by potassium chloride 0.15 M and Tris buffer 0.05 M, using an Ultra-turrax (3 $_x$ 15 s, 34000 revolutions per minute; at room temperature) with 50 ml tubes. Each sub-sample was transferred to another Pyrex-tube of 25 ml washing with 6 ml ethanol absolute (with 0.01% of 3,5-di-tert-butyl-4-hydroxytoluene (BHT)). In order to avoid early oxidation, 500 μl of pyrogallol was added. After that, each tube was closed and agitated by hand followed by the addition of nitrogen for 10 s. Next, the tubes were introduced in a water bath (70 ºC) for 5 min. Then,

nitrogen was added again for 10 s, followed by the addition of 5 ml potassium hydroxide solution (10 N, pH 7.4). Each tube was closed and agitated by hand. The tubes were again heated in a water bath (70 ºC) for 30 min. Finally, the tubes were cooled in ice for 10 min. Thereafter, the mixture was extracted three times with 4 ml of *n*-hexane:ethyl acetate 2:1 (v/v with 0.01% BHT). The supernatants, containing fat soluble vitamins, were transferred into another tube and subsequently, solvents were evaporated in a rotary evaporator (ILMVAC, Germany). Finally, the extract was re-dissolved in 100 *µ*l Tetrahydrofuran (THF) and 900 µl ethanol absolute (with 0.01% BHT) and transferred to vials for the immediate HPLC analysis. All sample preparation steps were carried out in a room with subdued light to prevent decomposition of the vitamins. For analysis of vitamins in feed the same procedure as for muscle was used with 2 g of sample. The concentrations of the individual vitamins were determined using a HPLC system (Shimadzu LC-10 AD, Japan). The system was 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*-, *δ*-, and *γ*-tocopherol; extinction: 295 nm, emission: 330 nm). Both detectors were used in series. The HPLC column used was a Synergy ODS, 250 $_{x}$ 40 mm (Phenomenex, USA). The Class-VP version 6.12 sp4 software was for evaluation of the chromatograms. The identification and quantification of the different vitamins was made by the use of an external standard procedure; the external calibration plots were made for a standard mixture in range of approximately 5–120 *µ*g/ml; the identification of the vitamins in the samples was made by the retention times of the standard compounds. The mobile phase was a mixture of acetonitrile and methanol (3:1 v/v) with a flow rate of 1.5 ml/min and a sample loop of 100 *µ*l.

2.6. Determination of trace elements

The determination of selected trace element contents (Se, Cu, Fe, and Zn) was performed on muscle tissue as recently described (Dannenberger et al., 2007). 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 apparatus (CEM, Kamp-Lintfort, Germany). The determination of the selected trace elements was conducted with an inductively coupled plasma mass spectrometer (ICP-MS 7500ce, Agilent Technologies, USA). Trace elements were analyzed twice for each sample and expressed as mg/kg of sample.

2.7. Statistical analysis

All data were analyzed by the least-squares means method using the GLM procedures of SAS®. The following model was used: $Y_{ij} = \mu + D_i + S_j + DS_{ij} + E_{ij}$, where Y_{ij} represents an observation; μ is the overall mean; D_i is the effect of ith diet (i = 1, 2, 3); S_j is the effect of jth storage time (j = 1, 2); DS_{ij} is the interaction between diet and the storage time and E_{ij} is the residual error. All tables contain the least squares means (LSMs) and the standard error of the mean (SEM). Significance differences, at $p \leq 0.05$, were determined using the Tukey test.

3. Results

3.1. Effect of diet on animal performance, meat quality, and composition

Animals from both treatment groups, fed grass silage and concentrate including rapeseed, had a lower metabolizable energy intake (MEI) (117.6, 97.1, and 87.9 MJ/day during the restricted period; and 122.8, 102.9, 104.1 during unrestricted period, for control, treatment group I and treatment group II, respectively) and a lower total daily live weight gain (Table 2.2) than control animals. Restricted animals (treatment group II) had the lowest daily gain during all the experiment, but the total daily gain was only significant against control animals. Animals from both treatment groups also had more days of fattening

and lower IMF (tendency, p = 0.1) than control animals. However, only restricted animals (treatment group II) were older at slaughter compared to the beef of the animals in the control group (Table 2.2). The tenderness was lower in restricted animals (treatment group II) than in unrestricted animals (treatment group I) and control animals. There were no significant differences for cooking loss and redness (a*), but color was lower and yellowness (b*) had a tendency to be lower (0.08) in the restricted animals compared with control animals (Table 2.2).

Diet caused significant changes in beef fatty acid composition, and there was no interaction between diet and storage (Table 2.3). Both treatment groups had higher percentages of sum PUFA, C18:1*trans*-11 (VA), C18:2*cis*-9,*trans*-11 (CLA), oleic acid (C18: 1*cis*-9), linolenic acid (C18:3*n*-3), eicosapentaenoic acid (EPA) C20:5*n*-3, docosapenteanoic acid (DPA) C22:5*n*-3 docosahexaenoic acid (DHA) C22:6*n*-3, sum *n*-3 FA, *n*-6/*n*-3, and lower AA/EPA (arachidonic acid/eicosapentaenoic acid ratio) than the control group. Linoleic acid, AA (C20:4*n*-6), and sum *n*-6 FA were only higher in unrestricted animals (treatment group I). Myristic acid (C14:0) and palmitic acid (C16:0) only decreased significantly in unrestricted animals. However, the sum of SFA did not change in a significant level.

Treatment groups showed a higher degree of lipid peroxidation at different times (15, 30, 60, and 120 min) than control animals (Fig. 2.1).

Table 2.2 Results of animal performance and meat quality parameters of *longissimus* muscle of German Simmental bulls.

		Treatment groups		
	Control group (n = 9)	I Unrestricted (n = 7)	II Restricted (n = 9)	p-Value
	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	
Initial age (d)	$283.67^{a}{}_{3.41}$	$270.14^{b}{}_{3.86}$	$277.11^{ab}{}_{3.41}$	0.0495
Initial weight (kg)	$295.11_{9.54}$	$288.86_{10.82}$	$284.00_{9.54}$	0.72
Weight at slaughter (kg)	$636.44_{4.11}$	$631.71_{4.66}$	$640.50_{4.11}$	0.38
Days of fattening (d)	$274.11^{a}{}_{10.84}$	$334.43^{c}{}_{12.29}$	$368.22^{bc}{}_{10.84}$	<0.0001
Age of slaughter (d)	$557.78^{a}{}_{12.82}$	$604.57^{ab}{}_{14.54}$	$645.33^{b}{}_{12.82}$	0.0003
Total daily gain (kg/day)	$1.26^{a}{}_{0.03}$	$1.03^{c}{}_{0.03}$	$0.97^{bc}{}_{0.03}$	<0.0001
Shear force at 14 d (kg)	$4.34^{a}{}_{0.62}$	$4.87^{a}{}_{0.71}$	$7.04^{b}{}_{0.62}$	0.01
Cooking loss (%)	$23.96_{1.06}$	$23.52_{1.20}$	$21.88_{1.06}$	0.36
Color *L 24 h**	$35.00^{a}{}_{0.68}$	$33.10^{ab}{}_{0.77}$	$32.50^{b}{}_{0.68}$	0.04
Color *a**	$17.65_{0.39}$	$17.57_{0.45}$	$18.13_{0.39}$	0.58
Color *b**	$2.99^{a}{}_{0.36}$	$2.14^{ab}{}_{0.41}$	$1.85^{a}{}_{0.36}$	0.08
IMF at 24 h	$2.20^{a}{}_{0.26}$	$1.55^{a}{}_{0.29}$	$1.54^{a}{}_{0.26}$	0.106

Means with different in the same row are statistically different ($p \leq 0.05$), LSM – least square means, SEM – standard error
IMF= intramuscular fat

Table 2.3 Fatty acid composition of *longissimus* muscle of German Simmental bulls at 24 h and 14 days after slaughter (g/100 g fatty acids, SumFA in mg).

	Diet							
		Treatment groups						
	Control group (*n* = 9) LSM_{SEM}	I Unrestricted (*n* = 7) LSM_{SEM}	II Restricted (*n* = 9) LSM_{SEM}	Storage		*p*- Value		
				LSM_{SEM}	LSM_{SEM}			
				24 h	14 d	D	S	D*S
C14:0	$2.55_{0.15}^{a}$	$1.91_{0.17}^{b}$	$2.33_{0.15}^{ab}$	$2.12_{0.12}$	$2.40_{0.12}$	0.02	0.12	0.86
C14:1	$0.48_{0.05}$	$0.37_{0.05}$	$0.43_{0.05}$	$0.44_{0.04}$	$0.41_{0.04}$	0.25	0.64	0.77
C16:0	$26.99_{0.62}^{a}$	$24.41_{0.73}^{b}$	$25.63_{0.62}^{ab}$	$24.48_{0.52}$	$26.80_{0.53}$	0.03	0.003	0.79
C16:1	$3.56_{0.15}^{a}$	$2.50_{0.18}^{c}$	$3.11_{0.15}^{ab}$	$3.46_{0.13}$	$2.68_{0.13}$	0.0001	0.0001	0.91
C18:0	$15.98_{0.35}$	$16.67_{0.41}$	$16.01_{0.35}$	$15.00_{0.30}$	$17.41_{0.31}$	0.39	<0.0001	0.62
∑ C18:1*trans*[c]	$1.55_{0.09}^{a}$	$2.08_{0.10}^{b}$	$2.11_{0.08}^{b}$	$2.01_{0.07}$	$1.82_{0.07}$	<0.0001	0.07	0.61
C18:1*trans*-11	$1.00_{0.07}^{a}$	$1.32_{0.09}^{b}$	$1.44_{0.07}^{b}$	$1.42_{0.06}$	$1.08_{0.07}$	0.0001	0.001	0.91
C18:1*cis*-9	$37.00_{0.80}^{a}$	$32.00_{0.90}^{b}$	$32.30_{0.80}^{b}$	$32.50_{0.70}$	$34.40_{0.70}$	<0.0001	0.04	0.52
C18:1*cis*-11	$1.37_{0.05}^{a}$	$1.47_{0.05}^{ab}$	$1.62_{0.05}^{b}$	$1.85_{0.04}$	$1.13_{0.04}$	0.001	<0.0001	0.07
C18:2*n*-6	$3.93_{0.73}^{a}$	$7.37_{0.87}^{b}$	$6.07_{0.73}^{b}$	$7.11_{0.63}$	$4.56_{0.64}$	0.01	0.005	0.36
C18:3*n*-3	$0.67_{0.13}^{a}$	$1.81_{0.15}^{b}$	$1.50_{0.13}^{b}$	$1.43_{0.11}$	$1.24_{0.11}$	<0.0001	0.22	0.55
C20:4*n*-6	$1.03_{0.26}^{a}$	$2.23_{0.31}^{b}$	$1.71_{0.26}^{ab}$	$2.32_{0.22}$	$1.03_{0.23}$	0.02	0.0001	0.30
C20:5*n*-3	$0.15_{0.06}^{a}$	$0.53_{0.07}^{b}$	$0.44_{0.06}^{b}$	$0.54_{0.05}$	$0.21_{0.05}$	0.0001	<0.0001	0.12
C22:5*n*-3	$0.35_{0.10}^{a}$	$0.88_{0.12}^{b}$	$0.74_{0.10}^{b}$	$0.91_{0.08}$	$0.41_{0.09}$	0.002	0.0001	0.21
C22:6*n*-3	$0.05_{0.02}^{a}$	$0.13_{0.02}^{b}$	$0.11_{0.02}^{b}$	$0.15_{0.01}$	$0.05_{0.01}$	0.004	<0.0001	0.12
CLA*c*-9,*t*-11*	$0.28_{0.01}^{a}$	$0.33_{0.02}^{b}$	$0.37_{0.01}^{b}$	$0.35_{0.01}$	$0.30_{0.01}$	0.0001	0.007	0.50
SFA[a]	$47.53_{0.77}$	$45.41_{0.91}$	$46.37_{0.77}$	$43.53_{0.66}$	$49.25_{0.68}$	0.21	<0.0001	0.44
MUFA[b]	$45.40_{0.85}^{a}$	$40.36_{1.00}^{b}$	$41.86_{0.85}^{b}$	$42.67_{0.72}$	$42.34_{0.73}$	0.001	0.81	0.49
PUFA[f]	$7.07_{1.35}^{a}$	$14.23_{1.60}^{b}$	$11.77_{1.35}^{b}$	$13.81_{1.16}$	$8.41_{1.18}$	0.004	0.002	0.35
Sum *n*-3[d]	$1.22_{0.30}^{a}$	$3.35_{0.35}^{b}$	$2.79_{0.30}^{b}$	$3.02_{0.25}$	$1.91_{0.26}$	<0.0001	0.003	0.29
Sum *n*-6[e]	$5.42_{1.07}^{a}$	$10.38_{1.26}^{b}$	$8.43_{1.07}^{ab}$	$10.24_{0.91}$	$6.06_{0.93}$	0.01	0.002	0.36
n-6/*n*-3	$4.39_{0.08}^{a}$	$2.94_{0.10}^{b}$	$2.94_{0.08}^{b}$	$3.59_{0.07}$	$3.26_{0.07}$	<0.0001	0.002	0.27
AA/EPA	$7.44_{0.14}^{a}$	$4.37_{0.16}^{b}$	$3.91_{0.14}^{b}$	$4.96_{0.12}$	$5.52_{0.12}$	<0.0001	0.002	0.20
SumFA	2710_{243}^{a}	1872_{286}^{b}	1838_{243}^{b}	1870_{207}	2378_{209}	0.02	0.08	0.87

LSM = least square means, SEM = standard error of LSM, D = effect of diet; S = effect of storage and D*S = interaction of diet *storage.

Means with different letter between treatments or between storage times in the same row are statistically different ($p \leq 0.05$).

* Coeluation with C18:2*trans*-7,*cis*-9 and C18:2*trans*-8,*cis*-10.

a 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.

b The sum of monounsaturated fatty acids (MUFA) was calculated as: C14:1 + C15:1 + C16:1 + C17:1 +PC18:1*trans* + C18:1*cis*9 + C18:1*cis*11 + C22:1 + C24:1.

c The sum of trans fatty acids (PC18:1*trans*) was calculated as the sum of the isomers C18:1*trans*-6–*trans*-11.

d 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.

e 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.

f PUFA is the sum of *n*-6 and *n*-3 FA.

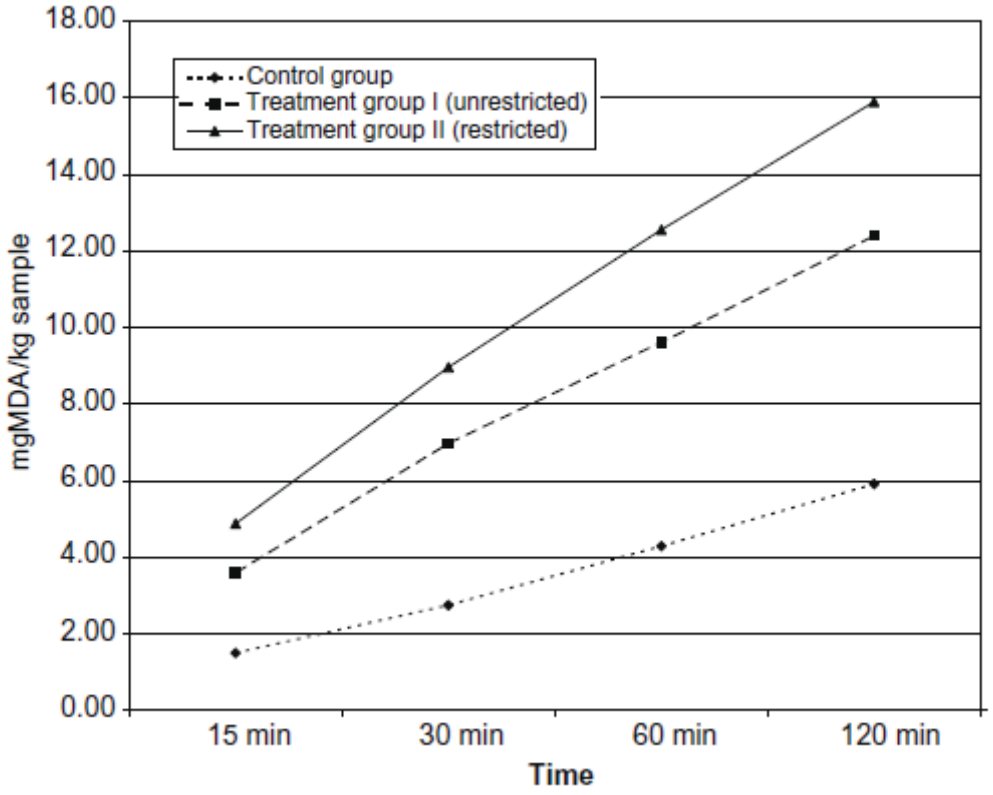

Figure 2.1 Lipid peroxidation of *longissimus* muscle of German Simmental bulls as measured by TBARS at different times. At 15 min $p = 0.0003$; at 30 min $p < 0.0001$; at 60 min $p < 0.0001$; at 120 min $p < 0.0001$. Differences between control group versus treatment groups.

Tocopherols and retinol were not significantly different between unrestricted animals from treatment group I and control group. Restricted animals from treatment group II had the lowest level of most vitamins without significant differences for most of them, except for γ-tocopherol that had a tendency to be lower than unrestricted animals (Table 2.4). The majority of trace elements were similar among groups ($p > 0.05$), except Se that was lower in both treatment groups (Table 2.5).

3.2. Effect of the beef storage

The total sum of fatty acids (SumFA) had a tendency to be higher (p = 0.07), and SFA proportions and the AA/EPA ratio were higher after 14 days. Instead, PUFA, sum *n*-3 FA, sum *n*-6 FA, and *n*6/*n*3 were lower (Table 2.3). Long chain PUFA were more affected with the storage. The vitamin concentrations were numerically lower after 14 days, but without significant differences (Table 2.4).

Table 2.4 Vitamin concentrations of *longissimus* muscle of German Simmental bulls at 24 h and 14 days after slaughter (mg/kg of sample).

	Diet			Storage				
		Treatment groups				*p*- Value		
Vitamins	Control group LSM_{SEM}	I Unrestricted LSM_{SEM}	II.Restricted LSM_{SEM}	LSM_{SEM} 24h	LSM_{SEM} 14d	D	S	D*S
Retinol (A)	$0.027_{0.007}$	$0.033_{0.007}$	$0.027_{0.006}$	$0.033_{0.005}$	$0.025_{0.006}$	0.76	0.29	0.90
δ –tocopherol	$0.024_{0.01}$	$0.042_{0.01}$	$0.029_{0.009}$	$0.037_{0.008}$	$0.026_{0.009}$	0.46	0.35	0.49
γ –tocopherol	$0.035_{0.01}^{a,b}$	$0.058_{0.01}^{a}$	$0.026_{0.008}^{b}$	$0.048_{0.008}$	$0.031_{0.008}$	0.06	0.15	0.73
***α* –tocopherol**	$0.936_{0.08}$	$1.042_{0.07}$	$0.927_{0.06}$	$0.992_{0.06}$	$0.945_{0.06}$	0.46	0.58	0.95
ß-Carotene	$0.174_{0.02}$	$0.177_{0.02}$	$0.157_{0.02}$	$0.172_{0.02}$	$0.167_{0.02}$	0.73	0.84	0.97

D = effect of diet; S = effect of storage; D*S = interaction of diet*storage.
Means with different letter between treatments or between storage times, in the same row are statistically different ($p \leq 0.05$).

Table 2.5 Selected trace element concentrations of *longissimus* muscle of German Simmental bulls at 24 h after slaughter (mg/kg of sample).

	Control group	Treatment groups		*p*- Value
		I Unrestricted	II Restricted	
	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	
Zn	$32.18_{1.11}$	$31.47_{1.26}$	$29.23_{1.11}$	0.18
Se	$0.12_{0.03}$ [a]	$0.104_{0.004}$ [c]	$0.106_{0.003}$ [bc]	0.003
Fe	$12.32_{0.59}$	$13.41_{0.67}$	$13.52_{0.59}$	0.31
Cu	$1.14_{0.18}$	$1.41_{0.20}$	$1.65_{0.19}$	0.15

Means with the different letter in the same row are significantly different ($p \leq 0.05$).

4. Discussion

4.1. Effect of diet

Cattle on the treatment diet failed to get similar daily live weight gain compared with control. It was associated to a lower MEI which also influenced the lower values observed for IMF. Likewise, there was no expected compensatory growth in restricted animals due to no strong increase in MEI after the restriction period. However, the results obtained offer important aspects to highlight the influence of diet and its restriction on beef quality and shelf life.

The treatment diet comprised of grass silage and concentrate including rapeseed (without restriction, treatment group I) resulted in similar tenderness and color in beef to the control diet based on a higher inclusion on maize silage and concentrate including soybean. These results contrast with results obtained previously (Dannenberger, Nuernberg, Nuernberg, & Ender, 2006) in German

Holstein and German Simmental bulls under a grass based system consisting of a period of summer pasture feeding followed by a winter indoor period on grass silage and a concentrate containing linseed, where beef was darker and tougher compared with bulls fed concentrate under indoor conditions. However, they agree with results of Keady, Lively, Kilpatrick, & Moss (2007) where the replacement of grass silage with maize silage (40:60) did not have an effect on tenderness and color. Cerdeno et al. (2006) and Warren et al. (2008) also found that muscle from grass-fed cattle does not always tend to be darker or tougher. Del Campo et al. (2008) reported that diet, animal age at slaughter and exercise could have an effect on meat color. These authors also reported that different external factors such as diet, pre-slaughter growth rate, animal age at slaughter, and the length of the finishing period, may affect beef tenderness. Since control animals and unrestricted animals from treatment group I, were under different diets, had different pre-slaughter growth rate, and had different days of fattening, but had similar indoor conditions and age at slaughter, it could indicate that the age at slaughter had more effects on beef color and tenderness. Therefore, there were no differences in these parameters. However, it is important to consider that a minimum feeding restriction, as practiced in this study for animals from treatment group II, could decrease significantly the beef color and especially the tenderness. This could be related to the significant increase in age at slaughter as mentioned before, and with changes in the anti-oxidative capacity during the restriction period. Savary-Auzeloux, Durand, Gruffat, Bauchart, & Ortigues-Marty (2008) found that total antioxidant capacity of muscles decreased during a restriction period and recovered when animals were re-fed *ad libitum*. These authors considered that a significant temporal decrease of the antioxidant status in vivo could be related to the protein oxidation process during meat maturation which needs to be more investigated.

The treatment diet allowed improvement of important parameters of beef fatty acid composition such as PUFA, CLA*cis*-9,*trans*-11, VA, *n*-3 FA, *n*-6/*n*-3 ratio,

EPA, DHA, and AA/EPA ratio, in both treatment groups. CLA and VA have been reported to have possible beneficial effects by reducing cancer in humans, considering in vitro results with human cells and animal models (Kelly, Hubbard, & Erickson (2007) and Miller, McHarth, Stanton,& Devery (2003), respectively). The *n*-6/*n*-3 ratio is also recommended as beneficial for different aspects related to human health, with maximum values of 4/1 (Simopoulos, 2008). In reference to the inflammatory processes, EPA and DHA are reported as very important fatty acids due to inhibition of AA metabolism to inflammatory eicosanoids. Instead, they increase mediators that are less inflammatory than those produced from AA or that are anti-inflammatory (Calder, 2008). For this reason, AA/EPA ratio has also been reported as very important for human health and considered as a new marker for human cancer (Garassino et al., 2006). In reference to SFA, there was a significant reduction of C16:0 and C14:0 in treatment group I (unrestricted). There is evidence that these fatty acids increase the risk of cardiovascular disease (Mozaffarian, 2008).

The treatment diet increased PUFA in beef and also increased the lipid peroxidation as measured by TBARS at different time of stimulation (15, 30, 60, and 120 min). These results agree with Corino et al. (2002) who evaluated TBARS in meat of pigs fed rapeseed after 300 min of induced peroxidation founding an increase of values over the time. However, our results contrast with results found by O'Sullivan et al. (2002), where beef from animals fed grass silage had lower lipid peroxidation than beef from animals fed maize silage or maize silage/grass silage (60:40) which was attributed to the higher *α*-tocopherol levels in the steaks from cattle fed grass silage. They also contrast with results obtained previously by Nuernberg et al. (2005) where beef from bulls under a grass-based system and a concentrate containing linseed, had lower lipid peroxidation than beef from bulls under an indoor concentrate system with maize silage. It was also related to the protection conferred by natural antioxidants present in grass. In this study beef vitamin levels were not significantly higher in the treatments groups, and Se was

lower in these groups. This could indicate the protection conferred by the treatment diet was not enough to compensate the higher offered PUFA levels. Possibly, the small differences in the amount of vitamins among diets of the control group and experimental groups, especially retinol and *α*-tocopherol, could influence the obtained results. Tocopherols and retinol are reported to be important antioxidants in beef (Descalzo & Sancho, 2008), because of an inverse relation between *α*-tocopherol concentration and lipid peroxidation in muscle (Wood et al., 2008).

Additionally, Daly, Moloney, & Monahan (2007) reported that not only the amount of *α*-tocopherol in beef is important, but also *α*-tocopherol/PUFA ratios, particularly the ratio of *α*-tocopherol to long chain PUFA such as EPA and DHA. In this case, both treatment groups increased EPA and DHA significantly, without changes in the same proportion of *α*-tocopherol. Likewise, Se is considered as an integral component of the antioxidant protection system of cells and tissues and has been recognized with antioxidant properties (Bobček et al., 2004) and with an inverse relation to TBARS in meat samples (DeVore, Colnago, Jensen, & Greene, 1983).

4.2. Effect of storage time

The increase of SFA proportion and the decrease of PUFA proportion after 14 days of storage are consistent with the results of previous studies in different species. In lean pigs, Stevez-Garcia & Cava (2004) found that SFA increased while the proportions of PUFA decreased in *longissimus* muscle after 10 days of refrigerated storage at 4 ºC. In pork, Nuernberg et al. (2006) also found that the percentages of linoleic acid, AA, EPA, and the sum of PUFA, especially *n*-6 fatty acids decreased after 6 days of storage at 5 ºC. However, Chen et al. (2007) evaluated the effect of storage on fatty acids composition of semitendinous muscle from Chinese cattle and found that beef had changes in only some fatty acids, but total PUFA, MUFA, SFA, *n*-3, and *n*-6 PUFA did not change after 10 days of

storage at 7 ºC. Likewise, Lazarus, Deng, & Watson (1977) did not find changes in the fatty acid composition of lamb during 9 days storage at 4 ºC. The differences between studies could be related to the amount of antioxidants present in meat before storage and to the balance in lipid peroxidation. Daly et al. (2007) reported that *α*-tocopherol concentration of approximately 3.5 *μ*g/g of fresh weight in beef *longissimus* muscle is sufficient to significantly stabilise lipids when stored under commercially relevant conditions. In this study, *α*-tocopherol concentration in beef was lower than 3.5 *μ*g/g. Additionally, all vitamins had lower levels after 14 days of storage (without significant differences), which is in agreement with Yang, Lanari, Brewster, & Tume (2002) and Pfalzgraf, Frigg, & Steinharth (1995), but contrary to Fredriksson & Pickova (2007). It is also supported by Rhee, Dutson, & Smith (2006) and Daly et al. (2007) who found an increase of lipid peroxidation in beef muscle during refrigerated storage. Then, the results obtained in this study could be explained by the increase in lipid peroxidation of PUFA during storage time. However, this cannot be confirmed since the lipid peroxidation (TBARS) was not measured after 14 days, which needs to be analysed in further investigations.

The results of this study demonstrated that a diet conformed by grass silage and concentrate including rapeseed, under unrestricted conditions, had a positive impact on beef quality. Detailed investigation is required to include the diet effects on the balance of the activity of the anti-oxidative system and lipid peroxidation in the different tissues of beef.

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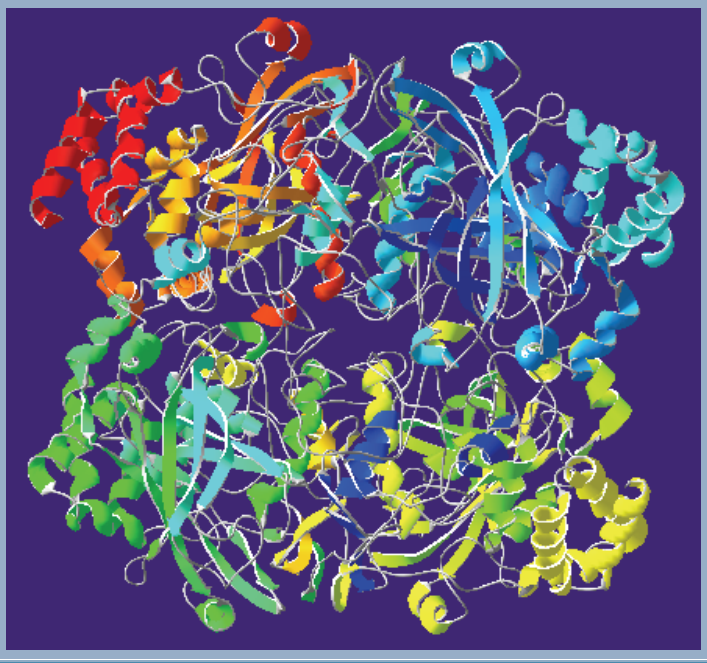

Chapter 3

Antioxidant enzyme activities and antioxidant capacity in longissimus dorsi muscle from bulls fed diets rich in polyunsaturated fatty acids

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Antioxidant enzyme activities and antioxidant capacity in *longissimus* dorsi muscle from bulls fed diets rich in polyunsaturated fatty acids

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Abstract

The effect of a treatment diet composed of grass silage and concentrate including rapeseed (with/without feeding restriction) was compared with a control diet of maize silage/grass silage (70:30) and concentrate including soybean, on the antioxidant enzyme activities of fresh *longissimus* muscle from German Simmental bulls. Additionally, the effect of diet on antioxidant capacity (AOC) of hydrophilic and lipophilic antioxidants was evaluated in fresh and stored beef muscle using the FRAP-ferric reducing ability and TEAC- Trolox-equivalent antioxidant capacity assays at different reaction times. Catalase and superoxide dismutase activities were significantly higher in the treatment diet groups, and glutathione peroxidase activity was not different. AOC was not affected by the diet. However, storage affected the values of FRAP and TEAC assays, and the results were time-depending. 30 minutes were found like a minimum reaction time for both assays.

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Generally, AOC values of the hydrophilic antioxidants were significantly higher than lipophilic values.
***Keywords*:** Catalase, Beef FRAP, Glutathione peroxidase, Superoxide dismutase, TEAC

1. Introduction

Since the last decade research is focused on improving the nutritional and health value of beef. Much attention has been given to strategies for increasing the content of *n*-3 polyunsaturated fatty acids (PUFA) (Scollan, Hocquette, Nuernberg, Dannenberger, Richardson, & Moloney., 2006; Mahecha, Nuernberg, Nuernberg, Ender, Hagemann, & Dannenberger., 2009). Additional interest is focused on the conservation of these PUFA for human consumption because PUFA are highly susceptible to lipid peroxidation by highly reactive species coming from endogenous and exogenous sources. Under balanced conditions, the body cells can minimize this oxidative damage due to their antioxidant defense conformed by non-enzymatic hydrophilic and lipophilic compounds and by endogenous enzymes like catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD). However, when the balance between the antioxidant defense and the highly reactive species is altered, lipid peroxidation increases quickly in beef tissues (Descalzo et al. 2007).

The most important method of manipulating the fatty acid composition of beef is by changing dietary ingredients which are known sources of long chain PUFA. These include forages and a range of oils and oil seeds (Scollan et al. 2006). Descalzo et al. (2007) demonstrated that the increase of *n*-3 PUFA contents in fresh beef from steers grazing pastures compared with diets based on grain silage is accompanied by a higher activity of some endogenous enzymes as well as by higher exogenous antioxidant contents, e.g. tocopherols. These antioxidants protect the PUFA in the tissues against free radicals attack. Similar results were

found by Gatellier, Mercier, & Renerre. (2004) in beef cattle grazed on pastures compared with animals fed a mixed diet composed of cereals mixture, silage, and cattle-cake of different origins. However, there is limited research comparing the overall antioxidant capacity (AOC) and antioxidant enzyme activities of beef cattle fed different diets, especially when they are supplemented at the same time with concentrate including rich sources of PUFA. Grass silage and other ensiled forages such as maize and whole-crop wheat have increased in popularity as a significant source of feed for cattle production in some countries of Europe (Mayne & O'Kiely, 2005). Therefore, there is a need for more research investigating the interactions of lipid peroxidation and antioxidant status in different tissues of beef cattle fed diets rich in PUFA.

Recently, different methods have been evaluated to assess AOC in beef muscle. Wu, Duckett, Neel, Fontenot, & Clapham (2008) evaluated ORAC assay (Oxygen Radical Absorbance Capacity); Min, Nam, Cordray, & Ahn (2008) evaluated FRC assay (Ferric ion reducing capacity); Descalzo et al. (2007) evaluated TEAC (Trolox-equivalent antioxidant capacity) and FRAP assays (ferric reducing ability); and Gatellier et al. (2004) evaluated ABTS (2,2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid]) and FRAP assay. To our knowledge, there is a lack of information about AOC of beef muscle using two different assays and to differentiate between the proportion of hydrophilic and lipophilic antioxidants of the overall AOC.

A study was established to investigate the effect of a treatment diet with higher level of *n*-3 fatty acids, composed of grass silage and concentrate including rapeseed (with and without feeding restriction) compared with a control diet with higher level of *n*-6 fatty acids, composed by a mixture of maize silage/grass silage (70:30) and concentrate including soybean on the quality and the interaction of lipid peroxidation and antioxidant status of muscle from German Simmental bulls. The diet effect on fatty acid profiles, micronutrients and lipid peroxidation in *longissimus*

muscle was described recently (Mahecha et al. 2009). The objective of this study was to evaluate the effect of diet on the antioxidant enzyme activities of fresh muscle and on AOC in fresh and stored muscle using FRAP and TEAC assay at different reaction times.

2. Material and methods

2.1. Animals and experimental design

The animals and treatments used in this study were previously described (Table 3.1, Mahecha et al. 2009). Twenty-five male German Simmental calves (3–4 months) were included in an indoor experiment comparing *n*-6 and *n*-3 PUFA enriched diets. *N*-6 PUFA enriched diets were considered as control and *n*-3 PUFA enriched diets as treatment diet. The animals were randomly assigned into three groups. The control group (n = 9) was daily fed maize silage/grass silage (70/30, *ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including soybean (2 kg the first 112 days, 2.5 kg the next 110 days, and 3 kg the rest of the time). Treatment group I, consisted of unrestricted animals (n = 7), fed grass silage (*ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including rapeseed cake in the same proportion as the control group. Treatment group II, consisted of restricted animals (n = 9), fed as treatment group I with a restriction of 1 kg of concentrate (50%) per day during the first 112 days of the fattening period (approximately 10 MJ/d of MEI). All groups included 5% of commercial mineral mix in the concentrate. Vitamins were not supplemented. All bulls were slaughtered at approximately 635 kg live weight by captive bolt stunning followed by exsanguination in the abattoir of the Research Institute for the Biology of Farm Animals in Dummerstorf (Germany). The slaughter and dressing procedures were in accordance with EU specifications.

Longissimus muscle samples were taken immediately after slaughter for enzymes activities and after 24 h for the measurements of AOC assays. Samples were taken at the 6th–13th rib of the right carcass side. One muscle slice (5 cm) was vacuum-packed and stored in the dark at 2 ºC for 14 days for AOC assays. After this time, it was kept at -20 ºC until the respective analysis.

Table 3.1 Fatty acid and micronutrients composition of the diets

Fatty acids (%)	TMR Control group	TMR Treatment groups
C4:0	0.14	0.19
C16:0	12.06	7.97
C16:1*cis*-9	0.44	1.43
C18:0	3.03	1.56
C18:1*cis*-9	23.07	35.60
C18:2*n*-6	46.58	26.96
C18:3*n*-3	7.25	8.06
PUFA	54.10	35.31
n-3 FA	7.36	8.21
n-6 FA	46.66	27.02
Ratio *n*-6/*n*-3	6.34	3.29
Vitamins (mg/kg)*		
Retinol (A)	13.71	14.79
δ –tocopherol	0.44	n.d.
γ –tocopherol	1.80	0.73
α –tocopherol	18.50	22.30
Trace elements (mg/kg)*		
Zn	24.70	25.50
Se	<0.01	<0.01
Fe	231.00	242.00
Cu	4.45	5.35

n.d. not detected
* mg/kg fresh material
Source: adapted from Mahecha et al. (2009)

2.2. Endogenous enzyme's activity

The muscle samples for endogenous enzymes were taken immediately after slaughter and stored at -70°C to prevent enzyme activity. The samples preparation started with frozen samples and during the whole preparation procedure the extracts were kept on ice-bath to prevent enzymatic reaction and peroxidation of unsaturated fatty acids. The samples were analyzed twice with one replicate.

2.2.1. CAT

CAT activity was measured according to the method described by Aebi (1974). The principle of this method is that hydrogen peroxide (H_2O_2) present in high amounts in a sample can be converted into water and oxygen by CAT. Then, to determine the CAT activity in samples, H_2O_2 is added in excess and the decrease of absorbance is measured by UV/VIS spectrophotometer. The rate of disappearance of H_2O_2 is directly proportional to the CAT activity and the concentration of H_2O_2 is considered a good estimation of it. Briefly, 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). Samples were homogenized by UltraTurrax (13000 rpm, ca. 45 sec). After centrifugation (7000 rpm, 20 min, 4 ºC), the supernatants were recovered and filtered over glass wool. Finally, 50 µL of the muscle extracts (kept in the ice-bath) were brought in a quartz cuvette (1 cm path length), and 2.9 mL of H_2O_2 solution were added. Immediately, the absorbance was monitored at 240 nm during 100 seconds using a Shimadzu UV-VIS spectrophotometer (PharmaSpec UV-1700). CAT activity was expressed in µmol x min-1 x g-1 (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 min.

2.2.1. GSH-Px

GSH-Px activity was measured according to the method described by Hernandez, Zomenno, Arinno, & Blasco (2004). The principle of this method is based on the GSH-Px function. GSH-Px reduces organic hydro peroxides to alcohols and water and also reduces H_2O_2 to water. Together with this reaction glutathione is turned into oxidized glutathione (GSSG). When GSSG reacts with NADPH, it is reversed again to GSH. Therefore, to determine the activity of GSH-Px, the amount of NADPH used to reduce GSSG to GSH is measured by UV/VIS spectrophotometry. The concentration of NADPH is considered directly proportional to the activity of GSH-Px. The supernatant fraction of the muscle homogenate described for CAT, was also used for the determination of GSH-Px activity. 300 µL of the muscle extracts (kept in the ice-bath), 2.65 mL of reaction solvent (1.13 mM), 0.57 mM EDTA, 1.13 mM NaN_3 (1.13 mM), 26 μL NADPH solution 17.3 mM, and 20 μL H_2O_2 solution were given in quartz cuvettes (1 cm path length). The absorbance was monitored at 340 nm during 400 seconds using a Shimadzu UV/VIS spectrophotometer (PharmaSpec UV-1700). The extinction coefficient of 6.220 μL μmol^{-1} cm^{-1} for NADPH at 340 nm and 25°C was used for the calculation. GSH-Px activity was expressed as μmol of oxidized NADPH μL^{-1} min^{-1} g^{-1} (U/g). One unit of GSH-Px activity was defined as the amount of extract required to oxidize 1 μmol of NADPH per min at 22 ºC.

2.2.1. *SOD*

SOD activity was measured according to the procedures of Marklund & Marklund (1974) using inhibition of pyrogallol autoxidation. SOD is an anti-oxidative enzyme that reduces superoxide anion radicals to H_2O_2 and oxygen. SOD activity was measured by the inhibition of pyrogallol autoxidation. In this indirect assay a unit of enzyme activity is defined as the amount of sample needed to inhibit the

reaction by 50%. The same sample preparation described for CAT determination of muscle homogenates was used for the determination of SOD activity. Six different amounts of muscle extracts were prepared for each sample 0, 10, 20, 30, 40, 50 μL extract and adding 175, 165, 155, 145, 135, and 125 μL distilled water, respectively. In each cuvette 2.75 mL of Tris-cacodylic buffer (pH= 8.2, 50 mM with DTPA) and 75 μL pyrogallol solution were added to start the reaction. The absorbance was monitored at 420 nm during 300 seconds using a Shimadzu UV/VIS spectrophotometer (PharmaSpec UV-1700). SOD activity was expressed as U/g of sample. One unit was taken as the SOD activity that inhibits the reaction by 50%.

2.3. Antioxidant capacity (AOC)

AOC was determined by the use of two different assays: Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC). The 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, according to Linden, 2003) to cover antioxidant capacity of both lipophilic and hydrophilic antioxidants in the muscle. These solvent systems were used to extract the hydrophilic and lipophilic antioxidants, respectively. Based on this, the methods are called $FRAP_{water}$, $FRAP_{lipid}$, $TEAC_{water}$, and $TEAC_{lipid}$ assays.

Approximately 5 g of frozen muscle samples were 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 20 414 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.

2.3.1. FRAP assay

The FRAP assay was carried out according to Benzie & Strain (1996). FRAP reagent solution consisted of 300 mmol/l acetate buffer (pH 3.6), 10 mmol/l TPTZ (2,4,4-Tri(2-pyridyl)-s-triazine, ≥99%, Fluka, Buchs, Switzerland) in 40 mmol/l hydrochloric acid, and 20 mmol/l iron (III)-chloride hexahydrate at the ratio of 10:1:1. 2950 µl of a fresh prepared reagent solution were mixed with 50 µl of the muscle extract. Extinction was measured at 593 nm (UV/Vis-spectrophotometer DU 530, Beckmann, Munich, Germany) after reaction times of 5, 30, and 60 minutes. The extinction of the sample at each reaction time was corrected with the blank and this value was called FRAP-1.

In order to correct for the endogenous iron content of the muscle samples, the FRAP assay without the addition of iron (III)-chloride solution was used according to Descalzo et al. (2007) with slight modifications. 300 mmol/l acetic acid solutions was mixed with a 10 mmol/l TPTZ solution in 40 mmol/l hydrochloric acid and distilled water at a ratio of 10:1:1. 2950 *µ*l of this reagent solution were mixed with 50 *µ*l of the muscle extract and extinction was measured after 5 min reaction time. The obtained extinction was corrected with the blank and this value was called FRAP-2. Finally, FRAP-1 was subtracted with FRAP-2 at each reaction time and the amount of reduced iron (final FRAP value) was determined by calibration of the method using known concentrations of iron (II)- sulfate-7-hydrate between 0.2-3.9 mmol/l Fe(II). FRAP antioxidant capacity was expressed as Fe^{2+} equivalents in *µ*mol/g muscle.

2.3.2. TEAC assay

In order to generate the ABTS radical, a stock solution of 8 mmol/l ABTS (2,2'-Azino-bis(3-ethylbenzo-thiazoline-6-solfonic acid) diammonium salt (Sigma, St. Louis, USA) and 3 mmol/l $K_2S_2O_8$ (potassium peroxodisulfate, Fluka, Buchs, Switzerland) was prepared by dissolution in water and incubation in the dark over night at room temperature. Prior to use, the stock 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. 2950 μl of the ABTS solution were mixed with 50 μl of the muscle extract and the decrease of absorbance was measured after 5, 30, and 60 min. AOC was determined by calibration using known concentrations of Trolox between 0.08 and 1.9 mmol/l.
TEAC antioxidant capacity was expressed as Trolox equivalents in μmol/g muscle.

2.4. Statistical analysis

All data were analyzed by the least-squares means method using Proc MIXED of SAS ®**.** For the analysis of enzyme activities, the following model was used: $Y_{ij} = \mu + D_i + E_{ij}$, where Y_i represents an observation; μ is the overall mean; D_i is the effect of i^{th} diet (i = 1, 2, 3), and E_i is the residual error. For the analysis of $FRAP_{water}$, $FRAP_{lipid}$, $TEAC_{water}$, and $TEAC_{lipid}$, the following model was used: $Y_{ijkl} = \mu + D_i + S_j + D_iS_j + T_k + S_jT_k + D_iS_jT_k + E_{ijkl}$, where Y_{ijkl} represents an observation; μ is the overall mean; D_i is the effect of i^{th} diet (i = 1, 2, 3), S_j is the effect of storage (j = 1, 2), T_k is the effect of the reaction time at which the measurements were done (k= 1, 2, 3), D_iS_j is the interaction diet and storage, S_jT_k is the interaction storage and time, $D_iS_jT_k$ is the interaction diet, storage, and time, and E_{ijk} is the residual error. All tables contain the least squares means (LSMs) and the standard error of the mean (SEM). Significant differences, at $p \leq 0.05$, were determined using the Tukey test. Tendency was considered when $p \leq 0.1$. Confidence intervals and t-test were made only for enzymes activity.

3. Results

3.1. Endogenous enzyme activities

The activity of the endogenous enzymes CAT, GSH-Px, and SOD is presented in Table 3.2. Diet caused significant changes on CAT and SOD activity with higher values in the treatment groups fed with grass silage and concentrate including rapeseed ($p<0.05$). Results were similar by Tuckey and t-test. In contrast to CAT and SOD activity, there was no significant effect of diet on GSH-Px activity ($p=0.16$) measured by Tuckey test. (Table 3.2), but confidence intervals seems to show a tendency between control and restricted animals that was corroborated by t-test ($p=0.05$).

3.2. Antioxidant capacity assays (AOC)

AOC of *longissimus* muscle using TEAC and FRAP assays at 24 h and after 14 days are presented in Figures 3.1-3.4 and Table 3.3. When the effect of diet on AOC was analyzed separately on fresh muscle (24 hours) or on stored muscle (14 days) at the same reaction time (Figures 3.1-3.4), diet did not affect the post-mortem AOC of muscle measured by both assays (FRAP, TEAC). The result was the same when the effects of storage and time were considered inside the effect of diet (Table 3.3). However, storage had a significant effect on AOC and the results were different according to the used assay, with increased AOC in the TEAC assay and decreased AOC in the FRAP assay after 14 days of storage (Table 3.3, Figure 3.1-3.4). Reaction time also had a significant effect on AOC and the values for all assays and all solvent systems increased with time (Table 3.3). The effect of storage was time-depending, the interaction storage*time was significant (Table 3.3). Using $FRAP_{water}$ assay, AOC values did not change in any of the groups after 14 days of storage at a reaction time of 5 minutes. AOC was lower in stored muscle of restricted treatment animals at 30 minutes and in all groups at 60

minutes compared to 24h samples (Figure 3.1). Using $FRAP_{lipid}$ assay, AOC values did not change in any treatment after 14 days of storage compared to 24 h, neither at 5 nor at 30 minutes reaction time, (Figure 3.2). At 60 minutes AOC was lower in the control animals and in the restricted treatment animals, remaining stable in the unrestricted treatment animals (Figure 3.2).

Using the $TEAC_{water}$ assay, AOC values were higher in all treatments after 14 days of storage at the different times of stimulation (Figure 3.3). Using the $TEAC_{lipid}$ assay, AOC values did not change in any treatment at 5 minutes of reaction time, but were higher in all treatments at 30 minutes and 60 minutes except in the unrestricted treatment group (Figure 3.4). The interactions diet*time, and diet*storage*time were not significant (p>0.05), and there was a tendency for the interaction diet*storage in the $FRAP_{water}$ assay (Table 3.3).

Comparing the TEAC and FRAP assays it became obvious that the extracts exposed a higher ability to reduce the ABTS radical than to reduce Fe^{3+}. Both assays showed higher AOC values in the assay carried out with hydrophilic extraction solvent compared to lipophilic extraction solvent.

Table 3.2 Effect of diet on the activity of endogenous enzymes in *longissimus* muscle of German Simmental bulls (U/g)

Enzymes	Control group (*n*=9)	Treatment groups I Unrestricted (*n*=7)	Treatment groups II Restricted (*n*=9)	Treatment comparison	Confidence interval Lower	Confidence interval Upper
				Control *vs.* unrestricted animals	-46.41	-16.08
				Control *vs.* Restricted animals	-36.39	-8.02
CAT	$76.67_{4.84}{}^{a}$	$107.91_{5.48}{}^{b}$	$98.87_{4.84}{}^{b}$	Restricted *vs.* unrestricted animals	-24.21	6.13
				Control *vs.* unrestricted animals	-0.21	0.58
				Control *vs.* Restricted animals	**-0.02**	**0.72**
GSH-Px	$1.66_{0.13}$	$1.47_{0.14}$	$1.30_{0.13}$	Restricted *vs.* unrestricted animals	-0.56	0.23
				Control *vs.* unrestricted animals	-5.32	-1.40
				Control *vs.* Restricted animals	-4.51	-0.94
SOD	$8.15_{0.63}{}^{a}$	$11.51_{0.71}{}^{b}$	$10.92_{0.63}{}^{b}$	Restricted *vs.* unrestricted animals	-2.54	1.38

Means with different superscripts are significantly different at P≤ 0.05 (results from Tuckey test).

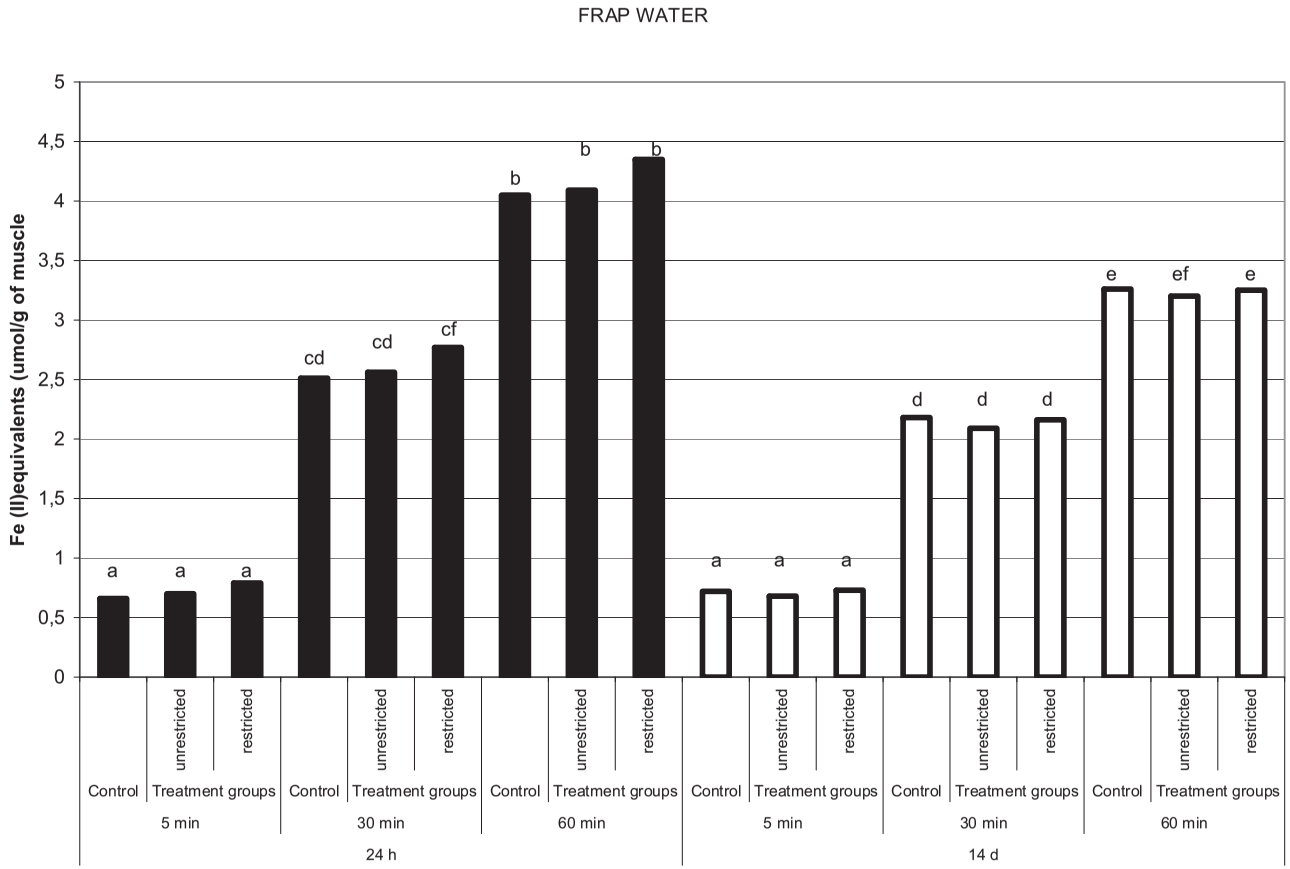

Figure 3.1 AOC measured by $FRAP_{water}$ assay for each treatment in different reaction times after 24 h post-mortem and after 14 days of storage. Different letters between bars indicate significant statistical difference $p \leq 0.05$.

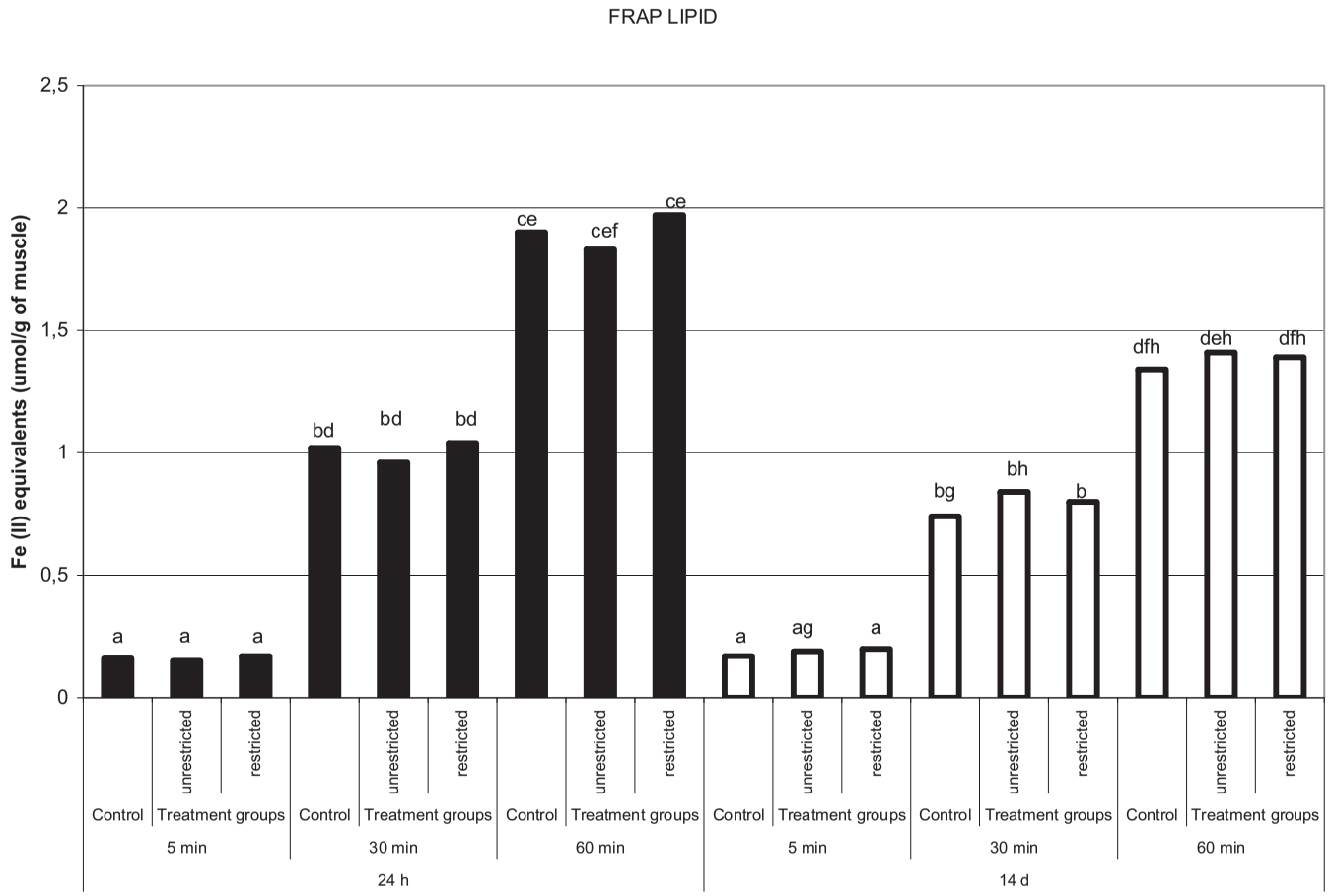

Figure 3.2 AOC measured by $FRAP_{lipid}$ assay for each treatment in different reaction times after 24 h post-mortem and after 14 days of storage. For footnotes see Figure 3.1.

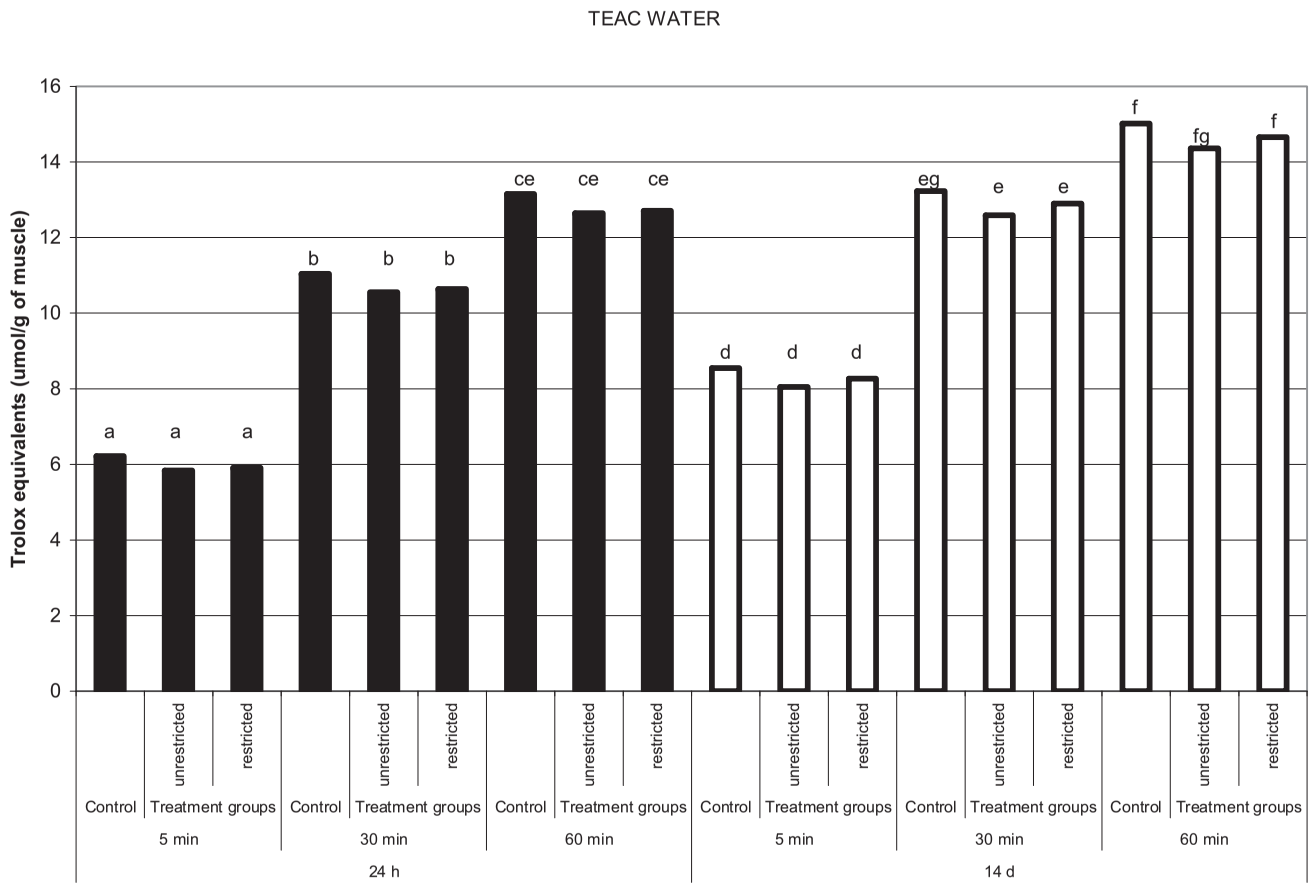

Figure 3.3 AOC measured by $TEAC_{Water}$ assay for each treatment in different reaction times after 24 h post-mortem and after 14 days of storage. For footnotes see Figure 3.1.

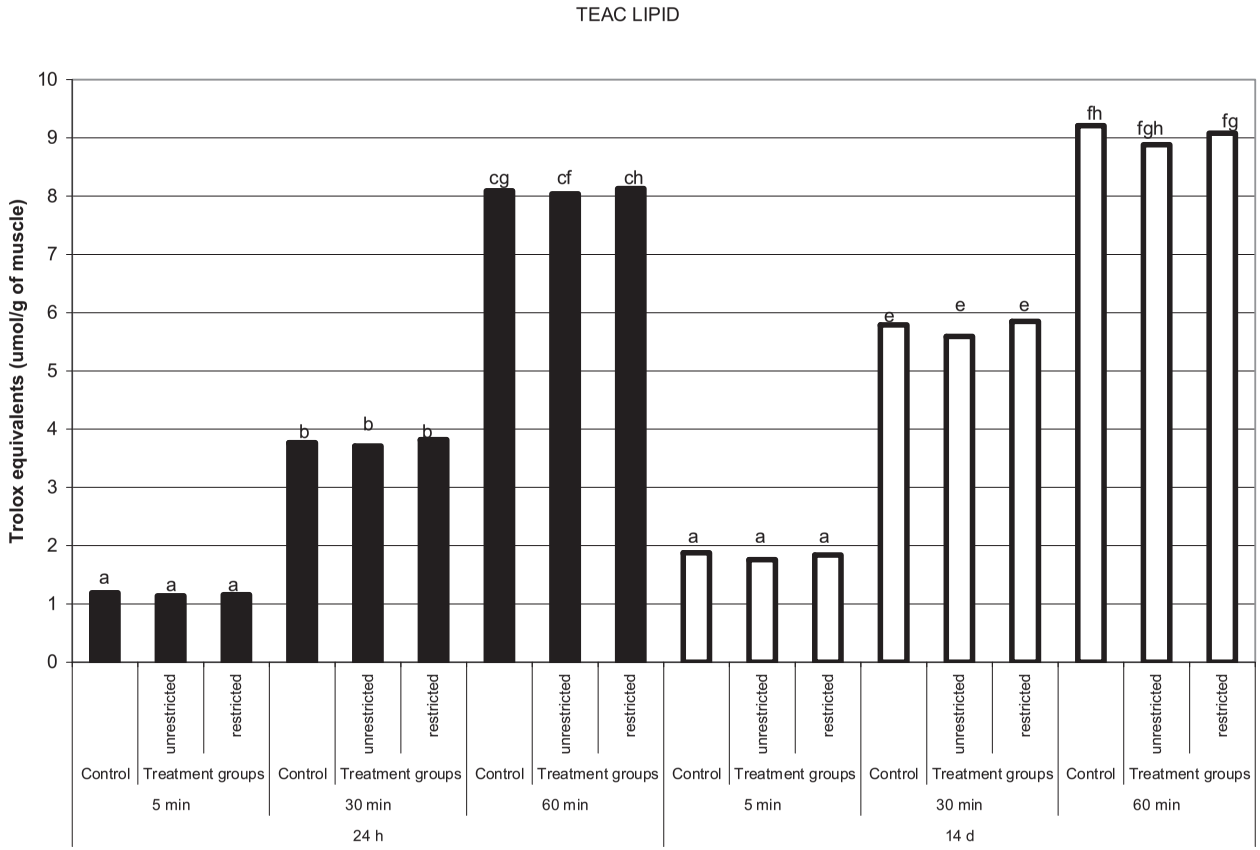

Figure 3.4 AOC measured by $TEAC_{lipid}$ assay for each treatment in different reaction times after 24 h post-mortem and after 14 days of storage. For footnotes see Figure 3.1.

Table 3.3 Effects of diet and storage on the total antioxidant capacity in *longissimus* muscle of German Simmental bulls

Assay	Control group	Treatment groups: I Un-restricted	Treatment groups: II Restricted	Storage: 24 h	Storage: 14 d	Reaction times: 5 min	Reaction times: 30 min	Reaction times: 60 min	P values: D	P values: S	P values: T	P values: Interactions D*S	D*T	S*T	D*S*T
	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	D	S	T	D*S	D*T	S*T	D*S*T
FRAP water	$2.23_{0.08}$	$2.22_{0.09}$	$2.34_{0.08}$	$2.50_{0.05}^{A}$	$2.03_{0.05}^{B}$	$0.71_{0.06}^{a}$	$2.38_{0.06}^{b}$	$3.70_{0.06}^{c}$	0.52	<0.05		0.08	0.96	<0.05	0.94
FRAP Lipid	$0.89_{0.06}$	$0.90_{0.08}$	$0.93_{0.06}$	$1.02_{0.04}^{A}$	$0.79_{0.04}^{B}$	$0.180.05a$	$0.90_{0.05}^{b}$	$1.64_{0.05}^{c}$	0.89	<0.05		0.58	0.99	<0.05	0.98
TEAC water	$11.20_{0.16}$	$10.67_{0.19}$	$10.85_{0.16}$	$9.86_{0.11}^{A}$	$11.96_{0.11}^{B}$	$7.14_{0.11}^{a}$	$11.82_{0.11}^{b}$	$13.76_{0.11}^{c}$	0.11	<0.05		0.58	0.97	<0.05	0.99
TEAC Lipid	$4.99_{0.16}$	$4.86_{0.19}$	$4.98_{0.16}$	$4.34_{0.11}^{A}$	$5.54_{0.11}^{B}$	$1.49_{0.12}^{a}$	$4.76_{0.12}^{b}$	$8.57_{0.12}^{c}$	0.85	<0.05		0.79	0.98	<0.05	0.99

This figure shows the combined effect of diet and storage on AOC, the comparison of AOC after 24 hours and after 14 days of storage including all treatments, the comparison of AOC at different reaction times including all treatments, and the interaction among effects. Means with different superscripts are significantly different at P≤0.05' (A,B for storage; a,b,c for reaction time)

FRAP values are expressed as Fe^{II} equivalent in mmol/g sample and TEAC values are expressed as trolox equivalents in mmol/g sample.

D: diet, S: storage, T: time, D*S: interaction diet * storage, D*T: interaction diet * time, S*T: interaction storage * time, D*S*T: interaction diet*storage*time

4. Discussion

4.1. Endogenous enzyme activity

The activity of CAT increased in muscle of bulls fed with grass silage plus concentrate including rapeseed. Literature reports different results about the effect of diet on CAT. Mercier, Gatellier, & Renerre (2004), Descalzo et al. (2007), Santé-Lhoutellier, Engel, & Gatellier (2008), and Insani, Eyherabide, Grigioni, Sancho, Pensel, & Descalzo (2008), did not found differences in the activity of CAT of muscle from cows, steers, lamb (castrated males), and steers fed with pasture or grain/concentrate, respectively. However, Gatellier et al. (2004) found higher CAT activity in pasture-fed heifers compared with animals fed a mixed diet composed of cereals mixture, silage, and cattle-cake of different origins, without difference in cows and steers. Likewise, Petron, Raes, Claeys, Lourenço, Fremaut, & De Smet (2007) found a tendency to increase in CAT activity of muscle from male lamb grazing pastures of different botanical composition. These results could probably indicate that diet has a different effect on CAT activity depending of the sex which needs to be investigated in deep. On the other hand, it has also been reported a higher CAT activity when the oxidative processes increase in the muscle (Renerre, Poncet, Mercier, Gatellier, & Metro, 1999). Turkeys fed diets supplemented with more or less unsaturated fat source (tallow/soy oil/rapeseed oil) showed a higher CAT activity in muscle when they were feeding with rapeseed oil. It was explained by a ‘‘feedback mechanism’’ as an answer to higher oxidative processes due to the higher amount of *n*-3 fatty acids (Renerre et al. 1999). In the present study, a higher lipid peroxidation (measured as TBARS) was found in treatments groups fed grass silage and concentrate including rapeseed which also had higher amount of *n*-3 fatty acids as described recently Mahecha et al. (2009) (TBARS values 1.5, 4.0, 3.6 at 15 min; 2.8, 9.0, 7.0 at 30 min; 4.3, 12.6, 9.6 at 60 min; 5.9, 15.9, 12,4 at 120 min, for control, restricted treatment, and unrestricted treatment animals respectively, with significant differences between treatments and control in all reaction times).

The higher activity of SOD found in treatment animals agrees with the results reported by Descalzo et al. (2007), Gatellier et al. (2004), Mercier et al. (2004), and Insani et al. (2008) in beef cattle fed pasture compared to mixed or grain diets. Contrary, Petron et al. (2007) and Santé-Lhoutellier et al. (2008) did not find differences between diets. Higher SOD activity in different tissues of beef cattle and lamb has been related to the higher amount of its main cofactors Cu (Jaramillo, Villa, Pineda, Gallego, Tabares, & Ceballos, 2005) and Zn (Nagalakshmi, Dhanalakshmi, & Himabindu, 2009) in diets. However, we do not have evidence to confirm this relation since the mineral differences between diets were not analyzed statistically in this study, and the obtained values of Zn and Cu in the treatment diets (25.50 mg/kg and 5.35 mg/kg, respectively) seem to be close to the control diet values (24.70 mg/kg and 4.45 mg/kg, respectively) (Mahecha et al. 2009). Nevertheless, considering that SOD and CAT are coupled enzymes (Descalzo et al. 2007), and that they showed the same pattern activity in this study, the answer in SOD could be explained by similar factors mentioned by CAT.

The effect of diet on GSH-Px in beef muscle was not clear. Contrary to CAT and SOD, the values of GSH-Px activity were lower in treatment animals, but there were no significant differences. However, t-test showed significant lower activity in restricted animals than in controls. Lower values in muscle of pasture-fed animals were found by Gatellier et al. (2004), Mercier et al. (2004), and Insani et al. (2008). However, Descalzo et al. (2007) and Santé-Lhoutellier et al. (2008) did not detect differences between diets. The differences between studies could be related to the amount of selenium (Se) in diets since GSH-Px is a seleno-dependent enzyme (Barciela, Herrero, García-Martín, & Peña., 2008). In this study, the differences in the Se concentration in the diets was no appreciable since the values were under the detection limit (<0.01 mg/kg), but treatment animals had lower concentration in muscle than control animals (Mahecha et al. 2009). Some studies have reported higher amount of Se in mixed diets compared to pasture diets (Gatellier et al. 2004). On the other hand,

the apparently effect of the feeding restriction on GSH-Px activity is not easy to compare since there is no enough studies about this effect. One of the last studies carried out in ruminants by Savary-Auzeloux, Durand, Bauchard, & Ortigues-Marty (2008) did not find effect of feeding restriction. Our results could be related to the higher lipid peroxidation found in the muscle of restricted animals compared to the controls (previous data reported by Mahecha et al. 2009). The skeletal muscle is considered particularly vulnerable to accumulative oxidative damage (Luhtala, Roecker, Pugh, Feuers, & Weindruch., 1994). It could also be associated to a negative effect of the feeding restriction on glutathione which is a specific substrate for this enzyme. A decrease in glutathione concentration after feeding restriction was reported in rats by Cho, Sahyoun & Stegink. (1981), and by Savary-Auzeloux et al. (2008) in lamb muscle.

4.2. Antioxidant capacity (AOC)

The results of the AOC measurements showed that diet did not affect the antioxidant capacity of the *longissimus* muscle from Simmental bulls using both assays (FRAP and TEAC), two extraction systems, and different reaction times (5, 30, and 60 minutes), in fresh or in stored samples. Our results confirm the results of Gatellier et al. (2004) and Descalzo et al. (2007). These authors found that AOC measured by TEAC and ABTS assays (respectively) was similar in beef muscle from steers fed pasture compared with animals fed grain or grain silage, respectively. In contrast, higher AOC values were found by Descalzo et al. (2007) in the muscle of animals fed pastures when AOC was measured by FRAP assay. Wu et al. (2008) also found significant differences between AOC values of muscle from beef cattle produced under different finishing systems, using ORAC assay with lipophilic extracts. However, they did not find differences with hydrophilic extracts. TEAC, ABTS, and ORAC assay are comparable methods that determine the radical scavenging abilities while FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. Considering that the results are different between studies using the same or different

assays, it could indicate that the effect of diet on AOC of beef muscle depends on the specific composition of each diet used. Wu et al. (2008) only found differences in AOC of muscle from beef cattle finished in a system based on alfalfa compared to a system based on high concentrate diets, but not between a system based on high concentrate diets and native pastures. It is important to consider that the muscle antioxidant defense is composed by non-enzymatic hydrophilic and lipophilic compounds (Descalzo et al. 2007) and AOC assays give a general idea of the quantitative contribution of all antioxidant substances to the antioxidant defense under the evaluated conditions. From this point of view and considering only the values of individual antioxidants measured in this study, no effect of diet either on lipophilic- nor on hydrophilic antioxidant activity would be expected since the analyzed vitamins did not show diet effects (retinol 0.03, 0.03, 0.03 mg/kg; α-tocopherol 0.94, 1.04, 0.93; mg/kg; β-carotene 0.17, 0.18, 0.16 mg/kg, for control, treatment group I, and treatment group II, respectively Mahecha et al. 2009). Likewise, GSH-Px activity was lower in muscle of treatment animals without significant differences while CAT and SOD muscle activity were higher in treatment animals. These results are opposite to other studies carried out with fresh pasture where it has showed higher levels of antioxidants compared to grain or concentrate (Descalzo et al. 2005; Gatellier et al. 2004). In this study, it was used grass silage instead of pasture. The differences could be explained by the loss of some antioxidants during the silage process. Thus, the treatment diet comprised of grass silage and concentrate including rapeseed had an important increase of *n*-3 fatty acids in beef muscle without the respective increase of the antioxidant defense. This unbalance resulted in an increased lipid peroxidation.

Additionally, our results demonstrated two observations: (a) storage and reaction time had a significant effect on AOC values, and (b) the effect of storage was time-depending. Thus, AOC values of stored samples from the same diet did not change after 5 minutes of reaction time, but it changed after 30 minutes by the $FRAP_{water}$ assay and after 60 minutes by the $FRAP_{lipid}$ assay. Similar time-depending results were found using TEAC assays. This could be

one explanation why contrary to our results, Descalzo et al. (2008) did not find changes in AOC of buffalo muscle measured by FRAP assay after 15 days of storage using only 4 minutes of reaction time. Furthermore, Min et al. (2008) neither found changes in AOC of beef, pork, and chicken breast measured by a similar method (Ferric ion reducing capacities FRC) after 7 days of storage. The last authors neither found changes in AOC of beef muscle measured by a method based on the free radical scavenging activities like TEAC. FRAP and TEAC assays consider that all antioxidants react quickly and that all reactions are finished within 4 and 6 minutes, but according to our results, the reaction times seem to be longer and depending on the individual antioxidants. Table 3.3 showed that antioxidants present in beef muscle continued reacting even at 60 minutes of reaction time in all assays. Additionally, results from the present study showed that some individual antioxidants react quickly at 5 minutes, others slowly until 30 minutes, and the others even until 60 minutes using both assays (Hubberman et al. in preparation). According to our investigation, at least 30 minutes are needed to determine AOC, using FRAP and TEAC assays in beef muscle samples. This corresponds to the evaluation about the chemistry behind antioxidant capacity assay, Huang, Ou, & Prior (2005) recommend a minimum of 30 minutes of reaction time for FRAP assay. The same recommendation is made by Zulueta, Esteve, & Frígola (2009) for TEAC assay in food products, by Wang et al. (2004) in plasma, and by Samaniego-Sánchez et al. (2007) in various olive oils. Furthermore, the results indicated a contrary effect of storage according to AOC assays being lower in FRAP assays and higher in TEAC assays after 14 days of storage. This may be explained due to different antioxidants that are reacting in each assay. Most probably the difference is mainly marked by some peptides and free amino acids, which can react in TEAC assay but not in FRAP assay. TEAC and FRAP assay using individual antioxidants revealed that glutathione, cysteine, and tyrosine reacted in TEAC assay strongly and in FRAP Assay hardly or not at all while ascorbic acid and NADH showed a higher activity in FRAP Assay (Hubberman et al. in preparation). Descalzo et al. (2007) showed that glutathione was unable to reduce Fe(III). Glutathione as well as small peptides such as carnosine and

anserine have been found in a higher amount in stored beef after 14 days of refrigerated storage (Bauchart et al. 2006). Similar increases can be found for different kinds of amino acids including cysteine and tyrosine (Koutsidis, Elmore, Oruna-Concha, Campo, Wood, & Mottram, 2008). Since carnosine, anserine (Gopalakrishnan, Decker, & Means, 1999), tyrosine, cysteine, phenylalanine, and histidine (Gebicki & Gebicki 1993) are reported as endogenous skeletal muscle antioxidants, the increase of them as a result of the tenderization process, and the higher response of TEAC assay to measure some of these peptides and free amino acids could be one explanation for the higher AOC values of stored muscle samples measured by TEAC assays. Moreover, the lower AOC values measured in stored samples using the FRAP assays could be probably related to the decrease of ascorbic acid. As it was mentioned before, ascorbic acid, as an individual antioxidant, showed a higher activity in FRAP Assay (Hubberman et al. in preparation). According to these results, FRAP assay is reflecting a loss of AOC in muscle after 14 days of storage considering the normal antioxidants present in muscle. These results is in agreement to the lower values of vitamins (without significant differences) and with the lower content of PUFA observed in treatment animals after 14 days (Mahecha et al. 2009) which could indicate a higher lipid peroxidation. Instead, TEAC assay is reflecting an increasing of AOC because considers amino acids resulting from the tenderization process that can have action as antioxidant and are not involved in FRAP assay. Additional information would be necessary in reference to lipid peroxidation after 14 days in order to clarify the useful of the increase of AOC observed by TEAC.

Our study has also revealed higher AOC levels found by both assays using hydrophilic than lipophilic extraction solvent systems, which could indicate that hydrophilic antioxidants such as ascorbic acid, ubiquinols, polyphenols, cellular thiols, and endogenous enzymes are present in higher amounts than lipophilic antioxidants, such as tocopherols and carotenoids. Higher AOC measured by ORAC assay using hydrophilic extraction solvent than lipophilic

extraction solvent was also obtained by Wu et al. (2008) in beef muscle from Angus-crossbred steers.

These results revealed that the discussion of diet effects on total antioxidant capacity is only valuable in combination with individual antioxidants and other parameters in beef muscle. Furthermore, the results confirmed the importance of using different methods to measure AOC values in beef samples as it has been mentioned by different authors, and to evaluate AOC in stored beef muscle samples by comparing different methods. A minimum of 30 minutes of reaction time in beef muscle samples is necessary using FRAP and TEAC assays. Additionally, the suitability of other AOC assays (e.g. photochemiluminescence) should be tested to describe better antioxidant status of beef. In future studies, more emphasis should be focused on the evaluation of high active antioxidants like beef ascorbic acid and glutathione composition relating to the overall AOC under different conditions. The evaluation of the contribution of endogenous antioxidant enzyme activity to the total AOC in beef muscle, and on the effect of feeding restriction on antioxidant enzyme activity in beef muscle should be included in these investigations.

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Chapter 4

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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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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Abstract

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

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1. 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 (Field, Blewett, Proctor, & Vine 2009). 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 (Ramsden, Faurot, Carrera-Bastos, Cordain, de Lorgeril, & Sperling, 2009). 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 (Scollan, Hocquette, Nuernberg, Dannenberger, Richardson, & Moloney, 2006). 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 (Simopoulos, 2008). Many studies have been conducted for the purpose of accumulating *n*-3 PUFA in beef (Scollan et al. 2006; Razminowicz, Kreuzer, Leuenberger, & Scheeder, 2008; Mahecha, Nuernberg, Nuernberg, Ender, Hagemann, & Dannenberger, 2009). 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 (Sante-Lhoutellier, Engel, & Gatellier, 2008). In

addition to FA composition, other factors influence the susceptibility of meat to lipid peroxidation (Tres, Bou, Codony, & Guardiola, 2008); specifically, an imbalance of antioxidant defenses to oxidative processes is the predominant factor that increases lipid peroxidation (Descalzo, Rossetti, Grigioni, Irurueta, Sancho, Carrete, & Pensel, 2007). 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 (Daly, Moloney, & Monahan, 2007; Warren, Scollan, Nute, Hughes, Wood, Richardson, 2008). In beef cattle, most research on this topic has focused on stabilizing PUFA with antioxidant supplements (Yang, Lanari, Brewster, & Tume, 2002; González, Bispo, Moreno, Monserrat, & Franco, 2008; Facco, Lage, & Godoy, 2009) or centered around the effect of different basal diets (pasture, grass silage, maize silage) sans PUFA enrichment (Insani, Eyherabide, Grigioni, Sancho, Pensel, & Descalzo, 2008; Descalzo et al. 2007; Gatellier, Mercier, & Renerre, 2004). 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 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.

2. Materials and methods

2.1. Animals and Diets.

A description of experimental animals and conditions has already been published for a subgroup of animals by Herdmann, Nuernberg, Martin, Nuernberg, & Doran (2010). 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 4.1). Bulls were slaughtered when reaching 623-630 kg live weight 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 ºC 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 ºC. All samples were taken from the 6th-13th rib of the right carcass side.

2.2. Fatty Acid Composition.

Lipids from 2 g of muscle were extracted with chloroform/methanol (2:1 v/v) according to the method of Folch, Lees, & Stanley (1956) by homogenization (Ultraturrax, 3 $_x$ 15 s, 12000 rpm) at room temperature. The FA composition of muscle lipids and feed was determined using the methodology described by Nuernberg, Nuernberg, & Dannenberger (2009). Briefly, the methodology involved capillary gas chromatography (GC) on a CP SIL 88, 100 m $_x$ 0.25 mm $_x$ 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 ºC, held for 5 min, increased to 200 ºC at a rate of 2 ºC min^{-1}, held for 10 min, and then increased to 220 at 1.5 ºC 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 ºC and the detector at 280 ºC. 19:0 methyl ester was used as internal standard.

Table 4.1 Chemical and Fatty Acid Composition of Total Mixed Ration (TMR)

	TMR for control group	*TMR for expetl 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 (% of FAME)		
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

2.3. Lipid Peroxidation.

To assess the amount of lipid peroxidation, a TBARS assay was used according to the methodology recently described by Mahecha et al. (2009). 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 ºC, 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 10000g 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.

2.4. 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. (2010). 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 20414g and 4 ºC 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 spectrophotometerUV-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 0 -azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and 3mmol/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).

2.5. Endogenous Enzymes.

The activity of antioxidant enzymes was measured as previously described (Mahecha et al. 2010). 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 ºC), the supernatants were recovered and filtered over glass wool.

Catalase (CAT). CAT activity was measured according to the procedures of Aebi (1974) 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 2.9mL 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 & Marklund (1974) 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 (Hernandez, Zomenno, Arinno, & Blasco, 2004). 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 (17.3mM), and 20 μL of H_2O_2 solution (22.5mM) 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 ºC.

2.6. 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. (2009). Briefly, three subsamples were prepared by homogenizing tissue (4 g per subsample) in 6 mL of a mixture composed by 0.15M potassium chloride and 0.05MTris buffer, using an Ultraturrax (3 $_x$ 15 s, 34000 rpm; at room temperature). The tubes were introduced in a water bath (70 ºC) 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 ºC) 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.

2.7. 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 (Mahecha et al. 2009, Dannenberger, Reichardt, Danier, Nuernberg, Nuernberg, & Ender, 2007). 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.

2.8. 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. (2005). Color was measured at three positions on one sample.

2.9. 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 (Malmo, 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).

2.10. 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 $_x$ 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.

3. RESULTS

3.1. 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 4.2).

3.2. 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 4.1).

3.3. Antioxidant Status.

3.3.1. 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 4.2). *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, $p = 0.001$).

3.3.2. 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 4.3). Diet did not show significant effects in muscle for the trace elements iron, selenium, copper, and zinc (Table 4.4).

Table 4.2 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.

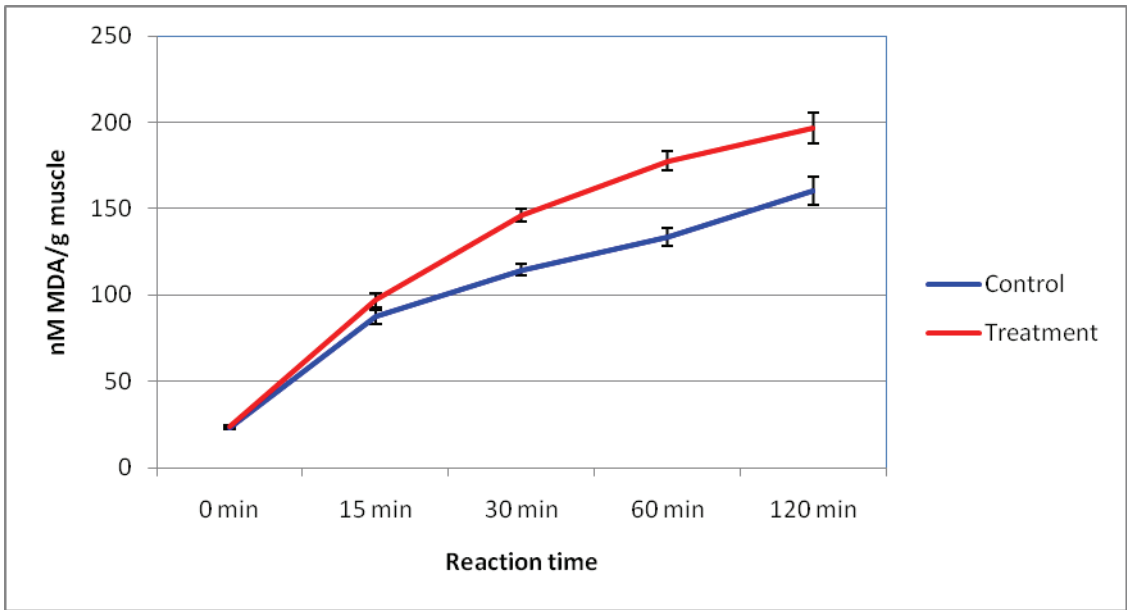

Figure 4.1 Lipid peroxidation of *longissimus* muscle from German Holstein bulls fed different diets as measured by TBARS at different times: at 0 min, $p = 0.71$; at 15 min, $p = 0.09$; at 30 min, $p < 0.0001$; at 60 min, $p < 0.0001$; at 120 min, $p = 0.01$.

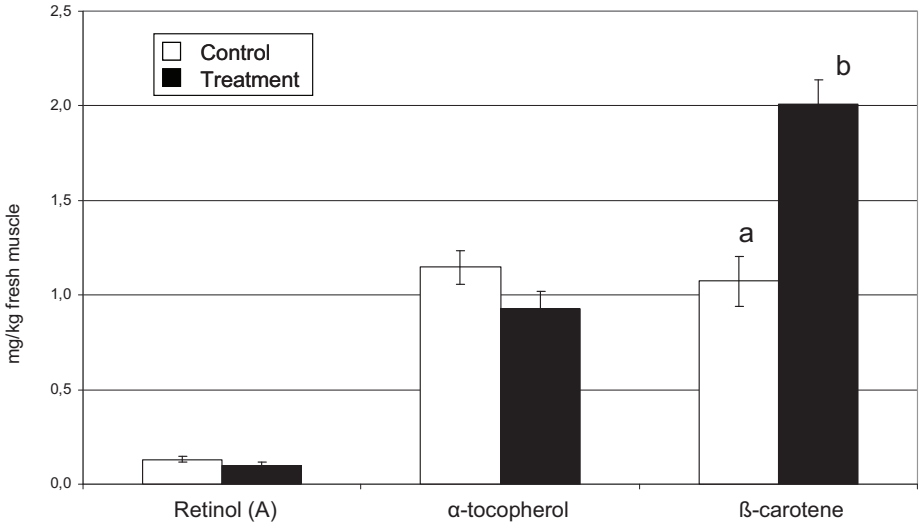

Figure 4.2 Vitamin concentrations of *longissimus* muscle from German Holstein bulls fed different diets: retinol (A), $p = 0.12$; α-tocopherol, $p = 0.09$; β-carotene, $p < 0.0001$. Means with different letters are significantly different at $p \leq 0.05$.

Table 4.3 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 4.4 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

3.3.3. 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 all assays (Table 4.5). There was no significant relationship between diet and reaction time.

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

3.4. Color.

There was no effect of diet on redness. However, lightness and yellowness decreased in treatment animals (Table 4.4).

Table 4.5 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	treatment	5 min	30 min	60 min			
	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	D	T	D x 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 x T, interaction diet x 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.

4. Discussion

4.1. 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 (Scollan et al. 2006, Razminowicz et al. 2008, Mahecha et al. 2009). 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 (Ramsden et al 2009, Simopoulos et al. 2008). 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 (Kohjima, Enjoji, Higuchi, Kato, Kotoh, Nakashima, & Nakamuta, 2009; Bergen, & Mersmann, 2005). However, in beef muscle, results from some studies using PUFA supplementation suggest a decrease (Pavan & Duckett, 2007), whereas others suggest an increase of de novo FA synthesis (Wistuba, Kegley, Apple, & Rule, 2007). 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 to the control diet enriched with *n*-6 PUFA (Table 4.2). 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 (Smith, Gill, Lunt, & Brooks, 2009; Shingfield, Rouel, & Chilliard, 2009). 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 0.17mg/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 (Nuernberg et al. 2005, Dhiman, Nam, & Ure, 2005). However, supplementing these diets with C18:2- or C18:3-rich plant oils has yielded varied results. Herdmann et al. (2010) 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 (De Smet, Raes, & Demeyer, 2004), 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 (Oka et al. 2002). 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 & Ntambi (2009). Likewise, Herdmann et al. (2010) related their observed tendencies with the presence of different observed isoforms for SCD.

4.2. 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 4.1). In a previous study (Mahecha et al. 2009), 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, Gatellier, & Renerre (2004) 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 56mg/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 (Gatellier et al. 2004), whereas others have found effects on only some of these parameters (Descalzo et al. 2007, Insani et al. 2008, Mahecha et al. 2010) or no effects at all (Sante-Lhoutellier et al. 2008). 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 GSHPx and SOD activities (Table 4.3). For GSH-Px, similar to the present study, Descalzo et al. (2007) 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 (Descalzo et al. 2007, Descalzo & Sancho, 2008). 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 ($O2^{\bullet-}$) by catalyzing its dismutation to H_2O_2 and molecular oxygen (O_2). The other antioxidant enzymes, CAT and GSH-Px, act to decompose H_2O_2 to water. According to Mete, Isik, Erdinc, & Gurkan (1999), 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. (2004) 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, Raes, Claeys, Lourenco, Fremaut, & De Smet (2007) 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 (Descalzo & Sancho, 2008). 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 2mg/kg of fresh muscle (Table 4.4), 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, Szopa, & Krasowska (2004) 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 (Gatellier, Hamelin, Durand, & Renerre, 2001). 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 non-radical products and may terminate radical reactions by binding to attacking free radicals (Paiva & Rusell, 1999). 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 (Gatellier et al. 2001). 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 4.5). Mahecha et al. (2010) also found an effect of diet on individual antioxidants; however, the antioxidant status of muscle from German Simmental bulls as measured by TEAC and FRAP assays was not significantly affected. Only Descalzo et al. (2007) 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. (2007), Gatellier et al. (2004) 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%.

4.3. Color Stability.

The color considerably influences beef's acceptability and purchasing decisions at retail points (Insani et al. 2008). 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 (Gatellier et al. 2004). In the present study, no differences were found in the redness of beef from animals fed either diet. Gatellier et al. (2001) found that the antioxidant *α*-tocopherol delays myoglobin oxidation and extends color stability on retail beef. Likewise, Yin & Cheng (1997) 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 (O'Sullivan, O'Sullivan, Galvin, Moloney, Troy, & Kerry, 2002), it is unclear if protein and lipid oxidation are concomitant processes or if one event precedes the other. In beef, Arnold, Arp, Scheller, Williams, & Schaefer (1993) demonstrated that lipid peroxidation and metmyoglobin formation occurred simultaneously but with different progression patterns at *α*-tocopherol levels of <3 *μ*g/g of muscle. However, no description has been reported that takes into consideration both *α*-tocopherol and *β*-carotene levels. Insani et al. (2008) 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 (Nuernberg et al. 2005, Gatellier, Mercier, Juin, & Renerre, 2005). This has been attributed to differences in ultimate pH values, sex, grade of oxygenation (Gatellier et al. 2005), age at slaughter, higher activity, and fat content (Yang et al. 2002). In the present study there were no differences between groups for pH (5.6 vs 5.6), sex, or activity. The differences in lightness

could be attributed to the lower fat content in muscle of treatment animals (2367 vs 1765 mg/100 g of muscle, control and treatment animals respectively, Table 4.2), age at slaughter, and possibly to the differences in oxidative conditions.

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, 2,20-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, catalase; SOD, superoxide dismutase; pyrogallol, 1,2,3-trihydroxybenzene; DTPA, diethylenetriaminepentaacetic acid; GSHPx, glutathione peroxidase; EDTA, Ethylenediaminetetraacetic acid; BHT, butylhydroxytoluene; HPLC; high-performance liquid chromatography; GLM, general linear model; LSM, least squares mean; SEM, standard error of LSM; CLA, conjugated linoleic acid; VA, vaccenic acid.

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Chapter 5

General discussion/conclusions and Thesis

5. General discussion/conclusions and Thesis

The farming and agricultural food industries including researchers are faced on an increasing demand by consumers for high-quality and safety products. One of the current major questions is how to increase the quality of animal products to guarantee these new requirements (Hocquette et al. 2009). In beef, a particular focus is given on the development of strategies to enhance the concentrations of those FA considered to be beneficial for human health, without causing a detrimental effect on the appearance, shelf-life or eating quality of the beef (Moloney et al. 2008). The main objective of this research was to evaluate the relationship of lipid peroxidation and antioxidant status including the influence of feeding *n*-3 and *n*-6 PUFA enriched diets on fatty acid composition and quality in *longissimus* muscle of bulls. In this chapter, results are discussed and concluded in a general way, and thesis and prospective research direction are exposed.

5.1. General discussion/conclusions

5.1.1. *Influence of feeding n-3 and n-6 PUFA enriched diets on fatty acid composition in longissimus muscle from bull*s

The information on fatty acid composition of muscle for the experiment 1 was presented as relative proportions and the effect of diet and storage was considered; it means that the values reflected the combined effect of diet in fresh and stored samples (Chapter 2). For the experiment 2, the concentration of FA (mg/100g sample) in only fresh muscle was included (Chapter 3). To discuss both experiments in a comparable way, a new Table was built including the results of the relative proportion (%) and concentration of FA (mg/100 g) in fresh muscle (Table 5.1).

One of the most important results obtained from both experiments is the improvement of the nutritional value of beef with the treatment diets, by favoring significant increase of the proportion and concentration of the sum of *n*-3 FA in muscle, especially the long chain *n*-3 PUFA (C20:5*n*-3, EPA, C22:5*n*-3, DPA, and C22:6*n*-3, DHA), and the decrease in *n*-6/*n*-3 ratio, with *n*-3 PUFA enriched diets despite of the high degree of biohydrogenation of dietary PUFA in rumen reported by Scollan et al. (2001). The recent scientific opinion from the European Food Safety Authority (EFSA) considered an intake for *α*-linolenic acid of 1-2 g/d consist with recommended intakes for individuals in the general population in some European countries based on considerations of cardiovascular health (EFSA, 2009). Likewise, EFSA (2010) recommended an intake for EPA + DHA of 250 mg/d in adults. Thus, considering that treatment diet in this study determined a content of *α*-linolenic acid and of EPA + DHA of 21 and 8.6 mg/100 g muscle, respectively, in the experiment 1 for unrestricted animals, and of 33.4 and 10.2 mg/100 g muscle, respectively, in experiment 2, it represents about 2 and 3% of recommended daily allowance for *α*-linolenic acid and for EPA+DHA, respectively, based on a serving size of 85 g of beef per day (31 kg of per capita consumption, Mir et al. 2004). Comparing the results of controls and treatment diets, the last one would allow an increase of 44% and 62% in the recommended daily intake of *α*-linolenic acid and EPA+DHA, respectively in the experiment 1, and of 157 and 113%, respectively in the experiment 2. As it was mentioned above, this result is important because of the relation of these FA to human health and their positive impact on the consumer acceptance of beef. According to Migdal et al. (2009), a very current important mark of beef quality is high level of long-chain *n*-3 PUFA (DHA, EPA, DPA). Then, the obtained results might have importance to give nutritional added value to the consumers contributing to market differentiation of beef.

Another important result was to find no significant changes in the proportion and concentration of SFA in experiment 1 or even to find no changes in the proportion and lower concentration of SFA in experiment 2 in animals fed with

treatment diets. The differences between experiments on SFA could be explained by the variation of data because in experiment 1, SFA also decreased with treatment diet, but differences were not significant. Results reported until now are no consistent and most of studies do not report both proportion and concentration of FA and it makes difficulties for the discussion. Kim et al. (2007) found a linear increase of SFA proportion in lamb of animals with the increase of *n*-3 FA in diet. Wistuba et al. (2007) found that SFA proportion in IMF of steers was increased with the inclusion of fish oil in the diet (rich in *n*-3 FA). Noci et al. (2007) found higher SFA by feeding linseed compared to sunflower oil. Nuernberg et al. (2005) found no differences in SFA proportion comparing bulls fed grass plus linseed oil (higher in *n*-3 PUFA) and bulls fed concentrate (higher in *n*-6 PUFA). Mach et al. (2006) found that SFA proportion did not change in bulls fed canola oil (diet higher in *n*-6 FA) compared with bulls fed linseed oil. Nuernberg et al. (1999) found that the higher incorporation of *n*-3 FA in polar muscle lipids of pigs resulted in a decrease of SFA. From our results, it seems that *de novo* SFA synthesis was negatively affected by the treatment diet in experiment 2 where *n*-3 PUFA were included in a higher concentration of diet. *De novo* FA synthesis involves two key enzymes, acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FAS). ACC carboxylates acetyl-CoA to form malonyl-CoA. The malonyl-CoA product is further converted by FAS to long-chain FA (Mashima et al. 2009). FAS (Joseph et al. 2010) and ACC (Zhao et al. 2010) are considered key rate limiting enzymes in the *de novo* pathway of fatty acid synthesis in muscle. The down-regulated expression of the lipogenic genes, as well as of the enzyme activity and protein expression means there is a reduced capacity for *de novo* synthesis of FA (Zhao et al. 2010). Herdmann *et al*. (2010a) working with a subgroup of animals from the same experiment of the present study did not find differences between animals fed *n*-3 and *n*-6 PUFA enriched diets on ACC protein expression in IMF. Instead, Ikeda et al. (1998) observed in adipose tissue of rats that linolenic acid inhibited FAS activity more efficiently than did linoleic acid. There is a lack of information on the differences of the effect of *n*-3 and *n*-6 PUFA on lipid metabolism in bovine muscle.

Most of results in bovine and rats have evaluated the effect of supplementation or not of dietary sources of linolenic acid (*n*-3 FA) and they have found decreased FAS expression and/or activity (Xu et al. 2008; Ikeda et al. 1998; Kim et al. 2003). According to Herdmann et al. (2010a), effects of experimental diets on ACC activity could take place without inhibition of ACC protein expression. One possible explanation for the decrease of SFA related to the *de novo* synthesis of FA found in the present study could be the increase of some intermediate FA coming from the rumen biohydrogenation, such as CLA*trans*-10,*cis*-12 and *trans*C18:1 isomers, which are known to exert anti-lipogenic effects in bovine tissues (Smith et al. 2009; Shingfield et al. 2010). The specific rumen condition developed with the treatment diet could have affected the production of some intermediate FA in rumen. In the present study the concentration of the sum of *trans* FA did not change in treatment animals of experiment 2, but the proportion increased. Likewise, although C18:1*trans*-10 isomer (Mahecha et al. 2010b) and CLA *trans*-10,*cis*-12 significantly decreased in muscle with the treatment diet, CLA *trans*-11,*cis*-13 increased (Herdmann et al. 2010c).

In reference to vaccenic acid (*trans*-11 C18:1, VA) and conjugated linoleic acid (C18:2 *cis*-9,*trans*-11, CLA), results can be summarized in **two main aspects**. **First**, there is an unclear effect of *n*-3 PUFA enriched diets on these FA, especially on CLA treatment diet of experiment 1, under *ad libitum* (unrestricted) conditions which failed to increase the proportion and concentration of VA and CLA in muscle. Instead, treatment diet of experiment 2 increased the proportion of VA and CLA, but without change in concentration of them. The proportion of CLA in ruminant products is dependent on the ruminal formation of both VA and CLA and on the tissue ability to desaturate VA into CLA (De la Torre et al. 2006). Although from both C18:2*n*-6 (linoleic acid) and C18:3*n*-3 (linolenic acid) is possible to get CLA for deposition in tissue (Figure 5.1), there is a lower metabolic efficiency to produce CLA from C18:3*n*-3 compared to 18:2*n*-6 (Bauman et al. 1999). From this point of view, diets higher in C18:2*n*-6 would be expected to increase CLA proportion in

muscle compared to C18:3*n*-3. However, it is known that conditions which increase VA in rumen can allow a higher flow of it from rumen to tissue to be transformed to CLA by the stearoyl CoA desaturase (SCD) (Dhiman et al. 2005). From the literature, it is known that basal diets containing a high proportion of grass or grass silage compared to concentrates resulted in a significant increased deposition of CLA in muscle (Enser et al. 1999; French et al. 2000). It also has been reported that diet based on pasture supplemented with sources rich in C18:2*n*-6 or C18:3*n*-3 compared to only pasture, increased proportion of CLA in muscle (Noci et al. 2007; Mir et al. 2003). Likewise, De la Torre et al. (2006) found a higher proportion of CLA when corn silage supplemented with linseed was compared to the same basal diet without linseed, but did not find differences when concentrate based diet was compared to the same basal diet supplemented with linseed. However, when the same basal diet is enriched with C18:2*n*-6 or C18:3*n*-3, the results are varied. Raes et al. (2004) did not find differences in muscle CLA of bulls comparing pasture supplemented with linseed *vs.* pasture supplemented with whole soybean; Kenny et al. (2007) did not find differences in muscle CLA concentration of bulls fed 1) 6% soybean oil (control), 2) 6% soybean oil + 1% fish oil, 3) 6% soybean oil + 2% fish oil, or 4) 8% palmitic acid for the first 50 days and 6% soybean oil + 2% fish oil for the second 50 days; and Noci et al. (2007) found higher proportion of CLA in muscle from animals fed pasture supplemented with sunflower oil *vs.* linseed oil. Different to Raes et al. (2004), Kenny et al. (2007), and Noci et al. (2007), the present study compared different basal diets enriched to *n*-6 or *n*-3 PUFA and found difference in CLA proportion, but not in concentration. In a previous study, Nuernberg et al. (2005) either found differences in CLA concentration when bulls were fed grass plus concentrate enriched in linseed oil (higher in *n*-3 PUFA) compared to bulls fed maize silage and concentrate enriched in *n*-6 PUFA. The increase in proportion, but not in concentration of CLA could be related to the decrease of the amount of fat in treatment animals. CLA concentration in the muscle of beef animals have not always been increased (Kenny et al. 2007) and there is an apparent paradox in that *n*-3 PUFA

supplementation enhances ruminal synthesis of VA, but then inhibits its conversion to CLA possibly through altering the activity of SCD. Waters et al. (2009) demonstrates that dietary *n*-3 PUFA inhibits expression of the gene that codes for SCD in bovine muscle tissue. Furthermore, they found evidence that the degree of inhibition of transcription for this gene is related to the level of dietary *n*-3 PUFA intake. In our experiment 2, it was found a lower activity of SCD in muscle of treatment animals (Herdmann et al. 2010c) which could have some effect on the desaturation of VA to CLA. Our results and results from literature suggest that the effect of the type of PUFA present in higher proportion in diet on beef CLA is not clear yet and as it was mentioned by Noci et al. (2007) clarification of the dietary ratio of C18:3*n*-3 to C18:2*n*-6 to maximize CLA synthesis continues being a focus point.

Second, there was a combined effect of diet and feeding restriction on the proportion of VA and CLA (Experiment 1). Thus, restricted treatment animals fed an *n*-3 PUFA enriched diet had a higher proportion of both FA compared to control animals fed *ad libitum n*-6 PUFA enriched diet. However, treatment animals fed *ad libitum n*-3 PUFA enriched diet had similar proportion of VA and CLA than control animals fed *ad libitum n*-6 PUFA enriched diet. Likewise, treatment animals fed the same treatment diet under restricted or unrestricted conditions did not have significant differences. There is a lack of information about the effect of bovine feeding restriction on the fatty acid composition of muscle and it is difficult to discuss this result, but a short-term food restriction in rats increased concentrations of some PUFA such as C18:2*n*-6, C20:4*n*-6, and C22:6*n*-3 in serum triglycerides and hepatic tissues (Chen & Cunnane 1992). Similarly, reducing energy supply during the finishing of Belgian Blue double-muscled cull cows reduced the proportions of SFA and MUFA and increased the proportion of PUFA in intramuscular fat; The low energy diet promoted the settlement of a discrete lipolytic state (Cabaraux et al. 2004). Results found by Hynes et al. (2003) showed an interactive effect of dietary fatty acid composition and energy restriction in rats

due to metabolic adaptation to energy restriction. Additionally, Moibi et al. (2000) mentioned that feeding restriction may cause diurnal variations in the rate of ruminal production. Nilsson et al. (2006) found that feeding restriction changed the rumen environment of reindeers getting higher pH (from 6.07 to 7.0, control *vs.* restricted). However, the restriction did not cause lasting effects on rumen function when the composition of the diet was not changed in the re-feeding period. Our results suggest that a combined effect of feeding restriction plus *n*-3 PUFA enriched diet could alter rumen environment leading an incomplete ruminal biohydrogenation of dietary fat increasing the proportion of VA and CLA for tissue deposition. More research is necessary on this topic. On the other hand, Lehnert et al. (2006) found an increase in SCD gene expression in bovine muscle of restricted animals. It may indicate that the muscle in the nutritionally restricted animals could have synthesized more CLA from VA as a specific response.

In Europe, beef normally is stored for approximately 14 days at 2-4 ºC in order to allow the transformation of muscle to meat (meat tenderization) and to get a better tenderness. Then, it was also considered important to know if the effect of the treatment diet could be conserved in stored samples for consumers. In the experiment 1, the fatty acid composition of fresh muscle taken after 24 h of slaughtering and stored muscle taken after 14 days of storage at 4ºC was compared. A summary of the main FA discussed in this document is presented as concentration in Table 5.2. For most of FA presented in this Table, the differences found between treatments in fresh samples were conserved in stored samples. However, when the average of all treatments were compared at 24 hours *vs.* 14 days to see the real effect of storage, EPA, DHA, and PUFA were significant lower after 14 days of storage; sum of *n*-3 FA, VA, and CLA did not change in a significant way; SFA had a significant higher concentration, oleic acid had a tendency for a higher concentration (p=0.07), and SumFA had a tendency for a higher concentration (related to a water loss during storage, p=0.08, Table 2.2). It indicates that with the treatment diet enriched in *n*-3 PUFA evaluated in experiment

1 compared to the control diet, is possible to offer a higher concentration of sum of *n*-3 FA, EPA, DPA, and DHA in fresh muscle, but only of sum of *n*-3 FA and EPA, in a lower level, in stored muscle. It is interesting how the concentration of CLA was conserved after 14 days of storage (comparing all treatments at 24h *vs.* 14 d). It could have importance in a future when CLA concentration can be significant increased with the diet just as it was gotten for *n*-3 FA. According to Dhiman et al. (2005) results from some studies suggest that CLA in meat is a stable compound under normal storage conditions. Shantha et al. (1994) found that the refrigerated storage, did not affect CLA concentration of ground beef. Likewise, results obtained by Rodríguez-Alcalá et al. (2007) did not find significant differences in CLA*cis*9,*trans*-11 of different CLA-fortified dairy products (milk, fermented milk, yogurt) under refrigerated storage for 1, 5, and 10 weeks.

Table 5.1 Fatty acid composition in fresh muscle of bulls from the experiment 1 and 2 in proportion (%) and concentration (mg/100g muscle)

	Experiment 1						Experiment 2			
	%			mg/100 g muscle			%		mg/100 g muscle	
	C	R	UR	C	R	UR	C	T	C	T
C14:0	$2.5_{0.2}^{ab}$	$2.2_{0.2}^{ab}$	$1.7_{0.2}^{b}$	$63.9_{11.3}$	$40.5_{11.3}$	$31.6_{12.8}$	$2.6_{0.1}$	$2.4_{0.1}$	$63.4_{6.0}^{A}$	$42.6_{6.3}^{B}$
C14:1	$0.5_{0.1}$	$0.5_{0.1}$	$0.4_{0.1}$	$14.7_{2.9}$	$8.0_{2.9}$	$6.9_{3.3}$	$0.66_{0.04}$	$0.62_{0.05}$	$16.4_{1.9}^{A}$	$10.9_{2.0}^{B}$
C16:0	$26.1_{0.9}^{ab}$	$24.4_{0.9}^{ab}$	$22.9_{1.0}^{b}$	$652.5_{102.8}^{AB}$	$422.2_{102.8}^{AB}$	$380.2_{116.6}^{B}$	$26.3_{0.4}$	$25.2_{0.4}^{(0.07)}$	$627.3_{52.0}^{A}$	$448.1_{53.9}^{B}$
C16:1	$4.0_{0.2}^{a}$	$3.6_{0.2}^{ac}$	$2.8_{0.2}^{bc}$	$103.1_{14.1}$	$60.7_{14.1}$	$46.4_{16.1}$	$3.7_{0.2}$	$3.3_{0.2}^{(0.07)}$	$89.2_{8.1}^{A}$	$57.9_{8.3}^{B}$
C18:1*cis*-9	$36.2_{1.1}^{a}$	$31.4_{1.1}^{b}$	$29.8_{1.2}^{b}$	903_{133}^{AB}	535_{133}^{B}	496_{151}^{B}	$37.1_{0.6}^{a}$	$34.7_{0.6}^{b}$	$892.8_{76.3}^{A}$	$614.9_{79.0}^{B}$
C18:1*trans*-11	$1.2_{0.1}^{ac}$	$1.6_{0.1}^{b}$	$1.5_{0.1}^{bc}$	$28.3_{4.6}$	$28.1_{4.6}$	$22.2_{5.3}$	$0.6_{0.0}^{a}$	$0.8_{0.0}^{b}$	$13.6_{1.2}$	$13.9_{1.2}$
C18:2*n*-6	$4.6_{1.0}^{a}$	$7.2_{1.0}^{ab}$	$9.6_{1.2}^{b}$	$95.4_{4.0}$	$96.9_{4.0}$	$92.1_{4.6}$	$5.3_{0.4}$	$5.6_{0.4}$	$112.9_{3.3}^{a}$	$95.2_{3.4}^{b}$
C18:3*n*-3	$0.7_{0.2}^{a}$	$1.6_{0.2}^{b}$	$2.0_{0.2}^{b}$	$14.6_{1.6}^{a}$	$22.8_{1.6}^{b}$	$21.0_{1.8}^{b}$	$0.6_{0.1}^{a}$	$2.0_{0.1}^{b}$	$13.0_{1.1}^{a}$	$33.4_{1.1}^{b}$
C20:5 *n*-3	$0.2_{0.09}^{a}$	$0.6_{0.09}^{bc}$	$0.8_{0.1}^{b}$	$3.9_{0.3}^{A}$	$8.2_{0.3}^{D}$	$6.5_{0.3}^{B}$	$0.2_{0.0}^{a}$	$0.5_{0.0}^{b}$	$3.8_{0.3}^{A}$	$8.8_{0.3}^{B}$
C22:5 *n*-3	$0.5_{0.1}^{ac}$	$1.0_{0.1}^{bc}$ (0.06)	$1.3_{0.2}^{b}$	$9.2_{0.3}^{A}$	$13.3_{0.3}^{C}$	$11.6_{0.4}^{D}$	$0.4_{0.0}^{a}$	$0.7_{0.0}^{b}$	$8.4_{0.3}^{A}$	$12.0_{0.3}^{B}$
C22:6 *n*-3	$0.07_{0.02}^{a}$	$0.17_{0.02}^{b}$	$0.2_{0.03}^{b}$	$1.4_{0.1}^{A}$	$2.3_{0.1}^{C}$	$2.1_{0.1}^{C}$	$0.05_{0.0}^{a}$	$0.08_{0.0}^{b}$	$1.0_{0.1}^{A}$	$1.4_{0.1}^{B}$
CLA*cis*-9,*trans*-11	$0.3_{0.02}^{ac}$	$0.4_{0.02}^{b}$	$0.3_{0.02}^{bc}$	$7.7_{1.2}$	$6.7_{1.2}$	$5.6_{1.4}$	$0.26_{0.01}^{a}$	$0.31_{0.01}^{b}$	$6.3_{0.7}$	$5.4_{0.7}$
Sum SFA	$45.4_{1.1}^{ab}$	$43.4_{1.1}^{a}$	$41.8_{1.2}^{a}$	1122_{175}^{AB}	747_{175}^{B}	673_{198}^{B}	$45.5_{0.6}$	$45.5_{0.6}$	$1078.7_{84.8}^{A}$	$805.5_{87.7}^{B}$
Sum MUFA	$46.2_{1.2}^{a}$	$42.2_{1.2}^{ab}$	$39.6_{1.4}^{b}$	1154_{167}	718_{167}	643_{188}	$45.1_{0.7}^{a}$	$42.5_{0.7}^{b}$	$1083.8_{97.1}^{A}$	$752.0_{95.3}^{B}$
Sum PUFA	$8.4_{1.9}^{ac}$	$14.4_{1.9}^{bc}$	$18.6_{2.2}^{b}$	$167_{7.8}^{AB}$	$189_{7.8}^{B}$	$175_{8.8}^{AB}$	$8.92_{0.64}^{a}$	$11.3_{0.68}^{b}$	$187.8_{5.6}$	$191.9_{5.8}$
Sum *n*-3	$1.4_{0.4}^{a}$	$3.4_{0.4}^{bc}$	$4.3_{0.5}^{b}$	$29.1_{2.2}^{A}$	$46.6_{2.2}^{B}$	$41.2_{2.5}^{B}$	$1.3_{0.1}^{a}$	$3.3_{0.1}^{b}$	$27.5_{1.4}^{A}$	$56.5_{1.4}^{B}$
Sum *n*-6	$6.5_{1.5}^{a}$	$10.4_{1.5}^{ab}$	$13.9_{1.7}^{b}$	$133_{5.1}^{AC}$	$138_{5.1}^{C}$	$131_{5.7}^{ABC}$	$7.5_{0.6}$	$7.7_{0.6}$	$157.6_{4.3}^{A}$	$131.5_{4.4}^{B}$
***n*-6/*n*-3 ratio**	$4.6_{0.1}^{a}$	$3.0_{0.1}^{b}$	$3.2_{0.1}^{b}$	$4.6_{0.1}^{A}$	$3.0_{0.1}^{C}$	$3.2_{0.1}^{C}$	$5.8_{0.1}^{a}$	$2.3_{0.1}^{b}$	$5.8_{0.1}^{A}$	$2.3_{0.1}^{B}$
SumtrFA	$1.7_{0.1}^{ac}$	$2.2_{0.1}^{b}$	$2.1_{0.1}^{bc}$	$40.1_{6.9}$	$38.5_{6.9}$	$31.7_{7.8}$	$1.3_{0.1}^{a}$	$1.4_{0.1}^{b}$	$30.3_{2.9}$	$25.2_{3.0}$
SumFA[h]				$2450.7_{311.8}$	$1660.9_{311.8}$	$1497.3_{353.6}$			2366.9_{182}^{A}	1764.5_{189}^{B}

C=control; R=restricted treatment animals; UR= Unrestricted treatment animals; T= treatment animals

SumFA[h]= sum of all identified fatty acids

a,b,c - Significant effect of the diet in % at $p \leq 0.05$; A,B,C,D - Significant effect of the diet in mg/100 g muscle at $p \leq 0.05$

Table 5.2 Fatty acid composition in fresh and stored muscle of bulls from the experiment 1 (in concentration)

	mg/100g muscle							
	Fresh muscle			Stored muscle			24 h	14 d
	C	R	UR	C	R	UR		
C18:1*cis*-9	903_{133}^{AB}	535_{133}^{B}	496_{151}^{B}	1117_{133}^{A}	681_{133}^{AB}	788_{163}^{AB}	645_{80}^{X}	862_{83}^{X} (0.07)
C18:1*trans*-11	$28.3_{4.6}$	$28.1_{4.6}$	$22.2_{5.3}$	$23.8_{4.6}$	$25.5_{4.6}$	$26.6_{5.7}$	$26.2_{2.8}$	$25.3_{2.9}$
C20:5*n*-3	$3.9_{0.3}^{A}$	$8.2_{0.3}^{D}$	$6.5_{0.3}^{B}$	$2.3_{0.3}^{C}$	$4.6_{0.3}^{A}$	$4.0_{0.3}^{A}$	$6.2_{0.2}^{X}$	$3.6_{0.2}^{Y}$
C22:5*n*-3	$9.2_{0.3}^{A}$	$13.3_{0.3}^{C}$	$11.6_{0.4}^{D}$	$6.2_{0.3}^{B}$	$8.4_{0.3}^{A}$	$7.6_{0.4}^{AB}$	$11.4_{0.2}^{x}$	$7.4_{0.2}^{y}$
C22:6*n*-3	$1.4_{0.1}^{A}$	$2.3_{0.1}^{C}$	$2.1_{0.1}^{C}$	$0.7_{0.1}^{B}$	$0.95_{0.1}^{AB}$	$0.83_{0.2}^{AB}$	$1.9_{0.1}^{X}$	$0.8_{0.1}^{Y}$
CLA*cis*-9,*trans*-11	$7.7_{1.2}$	$6.7_{1.2}$	$5.6_{1.4}$	$7.3_{1.2}$	$6.9_{1.2}$	$7.6_{1.5}$	$6.7_{0.8}$	$7.3_{0.8}$
Sum SFA	1122_{175}^{AB}	747_{175}^{B}	673_{198}^{B}	1483_{175}^{A}	1017_{175}^{AB}	1142_{214}^{AB}	848_{106}^{X}	1214_{109}^{Y}
Sum MUFA	1154_{167}	718_{167}	643_{188}	1353_{166}	853_{166}	969_{203}	838_{100}	1059_{104}
Sum PUFA	$167_{7.8}^{AB}$	$189_{7.8}^{B}$	$175_{8.8}^{AB}$	$157_{7.8}^{A}$	$169_{7.8}^{AB}$	$160_{9.5}^{AB}$	$177_{4.7}^{X}$	$162_{4.8}^{Y}$
Sum *n*-3	$29.1_{2.2}^{A}$	$46.6_{2.2}^{B}$	$41.2_{2.5}^{B}$	$29.4_{2.2}^{A}$	$42.0_{2.2}^{B}$	$41.8_{2.7}^{B}$	$39.0_{1.3}$	$37.7_{1.4}$
Sum *n*-6	$133_{5.1}^{AC}$	$138_{5.1}^{C}$	$131_{5.7}^{ABC}$	$117_{5.1}^{AB}$	$117_{5.1}^{AB}$	$108_{6.2}^{B}$	$134_{3.1}^{X}$	$114_{3.2}^{Y}$
***n*6/*n*3 ratio**	$4.6_{0.1}^{A}$	$3.0_{0.1}^{C}$	$3.2_{0.1}^{C}$	$4.0_{0.1}^{B}$	$2.8_{0.1}^{C}$	$2.6_{0.1}^{C}$	$3.6_{0.1}^{x}$	$3.2_{0.1}^{y}$

C=control; R=restricted treatment animals; UR= Unrestricted treatment animals
A,B,C,D - Significant effect of the diet in mg/100 g muscle at $p \leq 0.05$
x,y- Significant effect of storage in mg/100 g muscle at $p \leq 0.05$

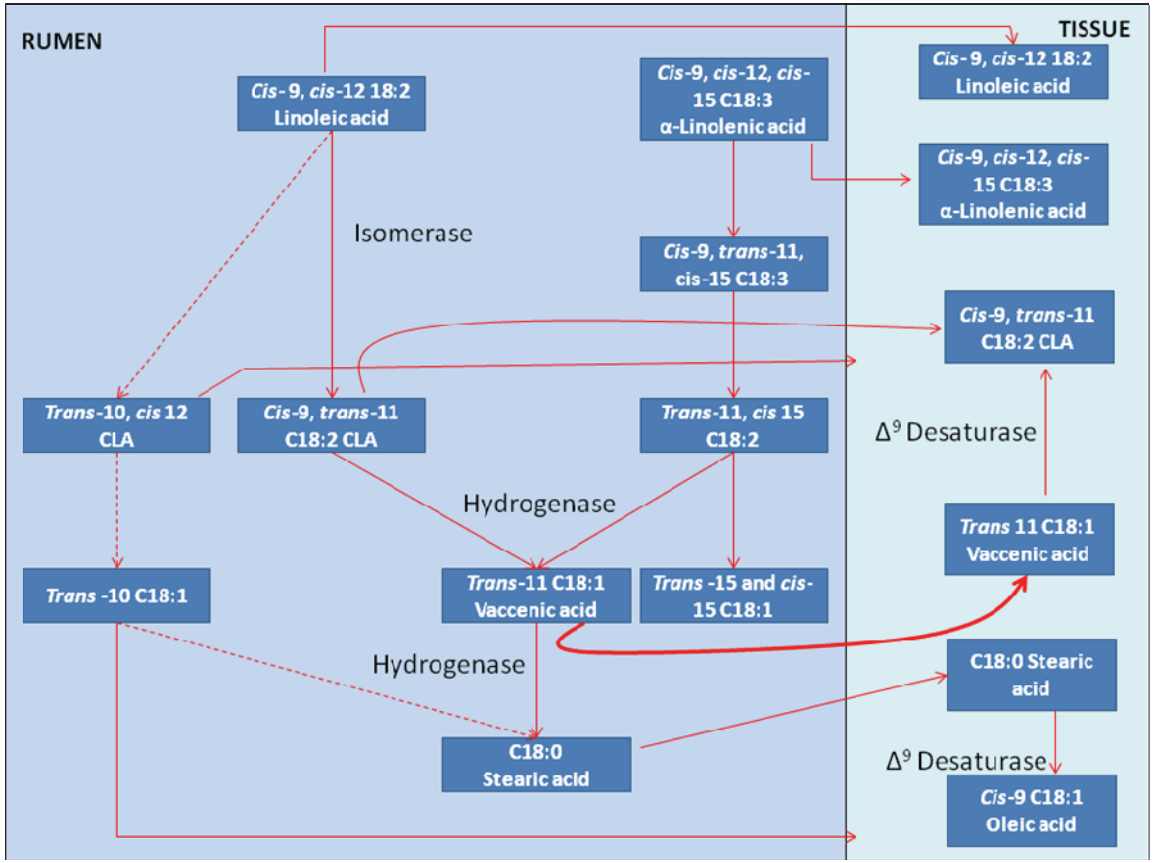


Figure 5.1 Biosynthesis of conjugated linoleic acid in ruminants.

Source: Adapted from Bauman et al. (2003).

5.1.2. Relationship between diets, lipid peroxidation and antioxidant status in longissimus muscle from bulls

In the relationship between lipid peroxidation and antioxidant status in muscle, it was possible to hightlight **three aspects**:

First, the treatment diets enriched in *n*-3 PUFA under *ad libitum* conditions increased muscle lipid peroxidation compared to control diets in both experiments, with important differences between experiments by increasing reaction times in which the increase of lipid peroxidation began to be significant. During TBARS measurements, samples react with a specific medium and the values are measured after 15, 30, 60, and 120 minutes of incubation. As the reaction times increase, the accumulation of reactive substances increases too. In the experiment 1, the treatment diet was enriched with *n*-3 FA on a lower level compared to the experiment 2, using different *n*-3 FA supplements. The control diet had 7.25 % C18:2*n*-3 *vs*. 8.06 % in treatment diet. Treatment diet increased the proportion and concentration of the sum of *n*-3 FA in muscle in 150 and 42%, respectively, with increase in the proportion and concentration (no significant) of PUFA (Table 5.1). Treatment animals presented significant higher lipid peroxidation than control animals at 15, 30, 60 and 120 minutes of reaction time. Bauchart et al. (2009) also found higher lipid peroxidation of muscle from cows when an increase of 56% in the proportion of C18:3*n*-3 was stimulated throughout the supplementation of extruded linseed. However, they did not find significant changes in the proportion of the sum of *n*-3 and PUFA. Our results indicate that the higher CAT and SOD activity, and the values obtained for the other measured antioxidants were not sufficient in muscle of treatment animals to balance the generated reactive substances. Additionally, treatment animals had significant lower concentration of selenium in muscle compared to control animal which could also affect the defense system. However, no significant decrease was found in GSH-Px activity. Although many of the benefits of selenium are related to its role in the activity of GSH-Px, it

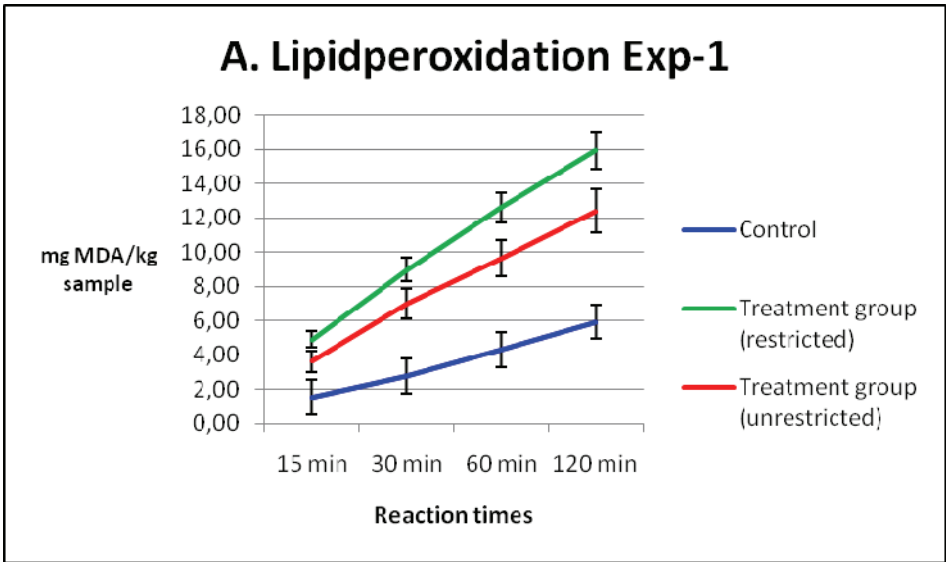
can also affect another key selenium-containing enzyme such as thioredoxin reductase (TR) which has not been studied in skeletal muscle. TR together with thioredoxin, forms a redox system with multiple roles, including the function of reducing ubiquinone-10 to regenerate the antioxidant ubiquinol-10 which is important in preventing peroxidation of lipidic substances such as α-tocopherol (Karunasinghe et al. 2006). The presence of TR has been reported from various tissues, including muscle (Rohrbach et al. 2006). In the experiment 2, control diet had 10.8 % C18:3*n*-3 *vs*. 35.5% in treatment diet. Treatment diet increased the proportion and concentration of the sum of *n*-3 FA in muscle in 154 and 105%, respectively, with increase in the proportion and concentration (no significant) of PUFA (Table 5.1). Treatment animals presented significant higher lipid peroxidation than control animals only from 30 minutes of reaction time. It suggests an appropriate antioxidant defense in treatment animals under low accumulation of reactive substances. In this way, results indicates that the higher concentration of β-carotene and CAT activity as well as the values obtained for vitamin A and α-tocopherol, and the activity of SOD and GSH-Px, were on a level to balance the reactive substances generated by the increase of *n*-3 FA in muscle under low oxidative conditions. β-carotene could have marked the difference. It had an increase of 92% in treatment animals compared to control animals. β-carotene could have an initial role as antioxidant under low accumulation of reactive substances and secondary role as pro-oxidant under high accumulation (Łukaszewicz et al. 2004; Paiva & Rusell, 1999).

Second, short time feeding restriction affected lipid peroxidation in muscle (experiment 1, Figure 5.2). Restricted treatment animals fed *n*-3 PUFA enriched diets had significant higher lipid peroxidation compared to controls fed *ad libitum n*-6 PUFA enriched diet, and there was a tendency for significant difference between restricted and unrestricted animals, fed the same diet, at 60 and 120 minutes of reaction time (*p* values= 0.10 and 0.11, respectively). Dietary energy restriction also tended to increase lipid peroxidation in *longissimus dorsi* muscle of pigs

(Mason et al. 2005). In the present study, it could not be associated to significant changes of endogenous enzymes or other antioxidants between restricted and unrestricted animals fed the same diet, but restricted animals fed *n*-3 PUFA enriched diet had lower GSH-Px activity than control animals fed *ad libitum* *n*-6 PUFA diet (p=0.05, t test). Results in rats showed a lower CAT activity in muscle of restricted animals (Filaire et al. 2009). However, Savary-Auzeloux et al. (2008) did not find effect of feeding restriction on GSH-Px activity in lamb. There are no data with regard to muscle in restricted bovines.

Third, the significant increase of some antioxidants in treatment animals from both experiments was not reflected on the total antioxidant capacity (AOC). As it was mentioned before AOC refers to a full spectrum of each component which shows antioxidant activity against reactive radicals in a special time point and in this way it gives a general idea of the quantitative contribution of antioxidant substances to the antioxidant defense in beef under the evaluated conditions. According to this, it looks like the balance in the quantitative contribution of antioxidant substances to the antioxidant defense was similar between control and treatment animals in both experiments. From the literature, it could be supported since studies that have found significant increase in most of individual evaluated antioxidants have also found significant differences in AOC measured by FRAP assay, but not by TEAC assay or a similar method (Descalzo et al. 2005, 2007; Gatellier et al. 2004). Results from this study also confirmed that the reaction between antioxidants present in the muscle samples and the reactive medium during the AOC measurements (using both assays and both extraction medium) continues even to 60 minutes after beginning with higher increase between 5 and 30 minutes. Then, a minimum reaction time of 30 minutes has been suggested for AOC measurements in beef muscle by using TEAC and FRAP assays. Other important result found in AOC was the differences between assays for stored samples (experiment 1). AOC values measured by FRAP assay were lower after 14 days of refrigerated storage while these values were higher by TEAC assay. This

difference was related to the different antioxidants that are reacting in each assay, especially some peptides and free amino acids, which can increase during the tenderization process and can react in TEAC assay but not in FRAP assay. FRAP assay does not measure thiol group -containing amino acids (Gupta et al. 2009). In the present study, TEAC and FRAP assay using individual antioxidants revealed that glutathione, cysteine and tyrosine reacted in TEAC assay strongly and in FRAP assay hardly or not at all (Hubberman et al. in preparation). Results of FA after 24 hours and 14 days of storage suggest a possible higher lipid peroxidation in stored samples since the concentration and proportion of PUFA, EPA and DHA were lower. Probably, these peptides and free amino acids that increase during the tenderization process can react as antioxidant, but not in a strong way. Thus, it was expected a lower or similar AOC in stored samples, but not a higher AOC as it was found by TEAC assay. Further studies are necessary to relate TBARS to AOC measured by TEAC assay in stored samples and in this way to determine the usefulness of TEAC assay in stored samples.



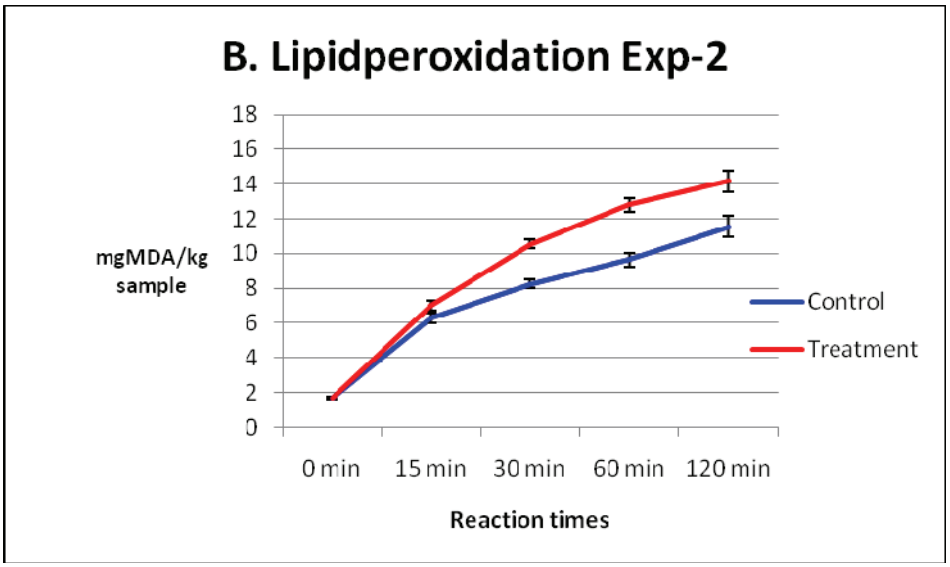


Figure 5.2 Lipid peroxidation of *longissimus* muscle from experiment 1 (A) and 2 (B). **A**. Lipid peroxidation of *longissimus* muscle of German Simmental bulls as measured by TBARS at different times. At 15 min $p=0.0003$; at 30 min $p<0.0001$; at 60 min $p<0.0001$; at 120 min $p<0.0001$. Differences between control group *vs.* treatment groups. **B**. 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 $p= 0.01$.

5.1.3. Influence of feeding n-3 and n-6 PUFA enriched diets on quality and its relationship to lipid peroxidation and antioxidant status in longissimus muscle from bulls

Three important aspects were obtained in the quality parameters from both experiments:

First, different *n*-3 PUFA enriched diets did not affect most of the measured quality parameters under *ad libitum* conditions, except muscle color which was affected when a high *n*-3 PUFA enriched diet was offered to treatment animals from experiment 2 (Table 5.3), but not when a lower increase in *n*-3 PUFA was offered in the treatment diet (experiment 1). Unrestricted treatments animals from experiment 1 need more time to get a similar weight at slaughter due to a lower total daily gain compared to control animals, but were slaughtered at similar age. The lower daily gain was related to a lower metabolic energy intake (MEI) which also decreased total fat content, although without significant differences. In spite of this, unrestricted treatment animals had similar quality parameters than control animals demonstrating that not always a diet including grass decrease muscle quality, specially tenderness and color which agree with Keady et al. (2007), Cerdeño et al. (2006), Warren et al. (2008). This also confirms the important influence of age to get similar parameters. On the other hand, this result indicates that the higher lipid peroxidation developed with the treatment diet of experiment 1 was not enough to affect muscle color in unrestricted animals. In the experiment 2, control and treatment animals got similar total daily gain, days of fattening, weight at slaughter, and MEI, but were slaughtered at different age. It was reflected in a similar quality parameters specially tenderness and the main color parameter redness (*a**). However, treatment animals had a lower lightness (*L**) and lower yellowness (*b**) than control animals. A lower lightness (*L**) was related to a significant effect of the treatment diet on muscle total fat content, age at slaughter, and oxidative conditions. In reference to yellowness, the obtained results were not

expected since a higher content of *β*-carotene has been linked to a higher yellowness. Lightness and yellowness values were significantly correlated with lipid levels in breast muscle of duck (+0.49 and +0.47, respectivelly) (Chartrin et al. 2006). Yellowness also decreased with fat content reduction of meat (Crehan et al. 2000). As treatment animals had lowered SumFA concentration (Table 5.1), it could affect the reduction of lightness and yellowness.

Second, the feeding restriction (experiment 1) affected tenderness. Restricted treatment animals had higher shear force than unrestricted one in spite of both groups were fed with the same diet and there were not significant differences in total daily gain, age at slaughter, pH, and time of fattening. Restricted animals under compensatory growth getting similar age at slaughter did not get differences in *longissimus* muscle tenderness compared to *ad libitum* animals (Therkildsen et al. 2008; Hansen et al. 2006; Purchas et al. 2002). Results are different in the present study and there are different conditions too. Restricted animals did not lose weight during the feeding restriction period compared to unrestricted animals and they did not compensate weight after this period either, resulting in similar total daily gain to unrestricted animals (Table 5.3). Although comparable results have been reported by Loerch (1990) in restricted steers with 20% or 30% of concentrate supplementation during the first 85 days of the growing period (246 kg of weight at the beginning), most of studies have found loses of weight during restriction and gain of weight after it (Therkildsen et al. 2008; Hansen et al. 2006; Purchas et al. 2002). Considering that the feeding restriction period was made during the first 90 days of the experimental period, the results found in the present study could be in part associated with an adaptation period for grass silage intake in both restricted and unrestricted animals which did not allow showing differences in daily gain during the restriction in favor of unrestricted animals. Tenderness is affected by different pre and post-mortem factors being post-mortem events, such as temperature, sarcomere length and proteolysis, which affect the conversion of muscle to meat, the main determinants (Maltin et al. 2003). However, it has been

suggested that variation in other factors that could be connected to the post-mortem proteolysis may also contribute to variation in meat tenderness, especially factors related to the calpain/calpastatin system. An increased calpain activity and reduced activity of its inhibitor calpastatin during the tenderization process is likely to be associated with a higher rate of post-mortem myofibrillar protein fragmentation which would enhance post-mortem tenderization of meat (Muir et al. 2001). However, regulation of calpain activity in muscle *in vivo* is not well understood and there is a lack of information about what is happening in the Calpain/calpastatin system of muscle *in vivo* during the feeding restriction and how it affects this system at different post-mortem times. A few results in different species are contrast (Goicochea & Conde, 1997; McDonagh et al. 1999; Muir et al. 2001). If calpain/calpastatin system activity is increased or inactivated during feeding restriction is not totally clear, but any change is affecting the final activity during the early post-mortem time or during the tenderization time which is affecting tenderness (Rhee et al. 2006; Kookmaraie & Geesink, 2006; Melody et al. 2004). Although in the present study it was not analyzed calpain/calpastatin system, literature suggests that calpain activity might have been affected during feeding restriction reducing its activity during the tenderization process and decreasing tenderness which needs to be research. In the same way, an increase (tendency p=0.1) in oxidative conditions in muscle of restricted animals compared to unrestricted animals fed the same *n*-3 PUFA enriched diet or to unrestricted control animals fed *n*-6 enriched PUFA diet (significant differences p<0.05), could have decreased post-mortem proteolysis and tenderness. Rowe et al. (2004) found that even small changes in oxidative conditions in meat can interfere with the tenderization process by suppressing calpain activity and slowing the rate of proteolysis occurring in meat during aging. Calpains are cysteine proteases; its activation requires the transfer of electrons between specific cysteine and histidine residues in its active site. For this, it is necessary that the cysteine and histidine residues be maintained in their properly charged state, a property that may be influenced by the microenvironment that is found around the enzyme, including the

redox state of the cell. The cysteine residue in the active site of calpains is highly susceptible to oxidation and to subsequent inactivation (Guttmann & Johnson, 1998). Savary-Auzeloux et al. (2008) also propose that changes in total antioxidant capacity of muscles in feeding restricted animals might be connected to proteolysis during the tenderization process. In the present study, there was only significant differences in GSH-Px activity between restricted and control animals.

Table 5.3 Animal performance and meat quality parameters of *longissimus* muscle of bulls from experiment 1 and 2

	Experiment 1			Experiment 2	
	C	R	UR	C	T
	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}	LSM_{SEM}
Weight at slaughter (kg)	$636.44_{4.11}$	$640.50_{4.11}$	$631.7_{14.66}$	$622.6_{6.3}^{\infty}$	$629.6_{6.5}^{\infty}$
Days of fattening	$274.11_{10.84}^{a}$	$368.22_{10.84}^{bc}$	$334.43_{12.29}^{c}$	$241.9_{10.2}^{\infty}$	$244.4_{10.6}^{\infty}$
Age at slaughter (d)	$557.78_{12.82}^{a}$	$645.33_{12.82}^{b}$	$604.57_{14.54}^{ab}$	$480.9_{9.85}^{A}$	$507.71_{10.2}^{B}$
Total carcass weight cold (kg)	$354.3_{2.9}^{\infty}$	$358.0_{2.9}^{\infty}$	$351.9_{3.2}^{\infty}$	$345.8_{4.1}$	$338.8_{4.2}$
LEM (kg)	$636.4_{4.2}$	$637.2_{4.3}$	$631.7_{4.9}$	$622.6_{6.3}^{\infty}$	$629.6_{6.5}^{\infty}$
Total daily gain (kg, g)	$1.26_{0.03}^{a}$	$0.97_{0.03}^{bc}$	$1.03_{0.03}^{c}$	$1230.0_{28.2}^{\infty}$	$1164.0_{29.2}^{\infty}$
Daily gain during FD (kg)	$1.47_{0.05}^{a}$	$1.03_{0.05}^{bc}$	$1.09_{0.05}^{c}$		
Daily gain after FD (kg)	$1.09_{0.04}$	$0.95_{0.04}$	$1.00_{0.05}$		
Liver (kg)	$7.27_{0.17}$	$7.14_{0.17}$	$6.60_{0.19}$	$8.0_{0.2}^{\infty}$	$7.6_{0.2}^{\infty}$
Heart (kg)	$2.6_{0.1}^{\infty}$	$2.6_{0.1}^{\infty}$	$2.6_{0.1}^{\infty}$	$2.7_{0.1}^{\infty}$	$2.7_{0.1}^{\infty}$
pH24h	$5.6_{0.0}^{\infty}$	$5.6_{0.0}^{\infty}$	$5.6_{0.0}^{\infty}$	$5.6_{0.0}$	$5.6_{0.0}$
Shear force 14 d (kg)	$4.34_{0.62}^{a}$	$7.04_{0.62}^{b}$	$4.87_{0.71}^{a}$	$4.13_{0.33}$	$4.59_{0.35}$
Color 24 h					
L*	$35_{0.68}^{a}$	$32.5_{0.68}^{b}$	$33.1_{0.77}^{ab}$	$33.2_{0.5}^{A}$	$31.2_{0.5}^{B}$
a*	$17.65_{0.39}$	$18.13_{0.39}$	$17.57_{0.45}$	$18.0_{0.4}$	$17.5_{0.4}$
b*	$2.99_{0.36}^{a}$	$1.85_{0.36}^{a}$	$2.14_{0.41}^{ab}$	$2.15_{0.3}^{A}$	$1.17_{0.3}^{B}$
Cooking loss	$23.96^{1.06}$	$21.88_{1.06}$	$23.52_{1.20}$	$20.03_{0.77}$	$20.30_{0.80}$
Muscle area (cm2)	$90.02_{3.79}$	$86.7_{3.79}$	$85.2_{4.29}$	$90.7_{4.7}^{\infty}$	$94.1_{5.1}^{\infty}$
Dry matter (%)	$25.9_{0.4}^{\infty}$	$24.5_{0.4}^{\infty}$	$25.4_{0.5}^{\infty}$	$26.3_{0.3}^{A\infty}$	$25.5_{0.3}^{B\infty}$
IMF (%)	$2.2_{0.26}$	$1.54_{0.26}$	$1.55_{0.29}$	$2.8_{0.3}^{\infty}$	$2.1_{0.3}^{\infty}$
Protein (%)	$22.2_{0.2}^{\infty}$	$21.7_{0.2}^{\infty}$	$22.2_{0.2}^{\infty}$	$22.5_{0.1}^{\infty}$	$22.4_{0.1}^{\infty}$
Ash (%)	$1.1_{0.0}^{\infty}$	$1.1_{0.0}^{\infty}$	$1.1_{0.0}^{\infty}$	$1.1_{0.0}^{\infty}$	$1.0_{0.0}^{\infty}$

$^{\infty}$ Published by Herdmann et al. (2010 b, c). FD= Feeding restriction

5.2. Thesis

This study investigated the influence of feeding *n*-3 and *n*-6 PUFA enriched diets on the fatty acid composition, oxidative stability, overall quality, and their interrelationships in *longissimus* muscle of bulls. Two experiments were performed under indoor conditions. The **Experiment 1** was carried out with **Simmental German bulls,** comparing control animals fed maize silage/grass silage (70/30, *ad libitum*), molasses, hay, and concentrate including soybean, with treatment animals fed grass silage, molasses, hay and concentrate including rapeseed under restricted and unrestricted conditions. The **Experiment 2** was carried out with **German Holstein bulls,** comparing control animals fed maize silage, and concentrate enriched with *n*-6 FA (soybean-based concentrate), with treatment animals fed grass silage, and concentrate enriched with *n*-3 FA (linseed oil and rapeseed cake based concentrate). The increase of *n*-3 FA in treatment diet was low in the experiment 1, and high in the experiment 2, compared to control diets. The results of this study allow extracting the following thesis:

1. The different *n*-3 PUFA enriched diets improved the fatty acid composition of *longissimus* muscle in reference to an increase of the sum of *n*-3 FA with lower or similar concentration of SFA which is considered beneficial for the human health.

2. There was a combined effect of *n*-3 PUFA enriched diet and feeding restriction on VA and CLA proportions of *longissimus* muscle.

3. The treatment diet enriched in *n*-3 PUFA evaluated in experiment 1 increased the concentration of sum *n*-3 FA, EPA, DPA, and DHA in fresh muscle, but only of sum *n*-3 FA and EPA, in a lower level, in stored muscle.

4. *N*-3 PUFA enriched diets from both experiments, under *ad libitum* conditions, developed higher lipid peroxidation in muscle compared to *n*-6 PUFA enriched

diets, and the effect was different at different reaction time which was related to the antioxidant status offered by each diet.

5. Short time feeding restriction affected lipid peroxidation in muscle.

6. Antioxidant capacity (TEAC and FRAP assay) was not affected by the diet. TEAC and FRAP assay had different answer in storage samples. TEAC values increased in stored samples while FRAP value decreased.

7. AOC values continuously increase in TEAC and FRAP assays even after 60 minutes after starting the stimulation of reaction having the highest increase until 30 minutes.

8. Different *n*-3 PUFA enriched diets did not affect most of the measured quality parameters under *ad libitum* conditions, except muscle color which was affected when a high *n*-3 PUFA enriched diet was offered to treatment animals from experiment 2.

9. Feeding restricted animals had significantly lower tenderness of muscle at 14 days of refrigerated storage compared to unrestricted animals feeding the same diet.

Chapter 6

Prospective Research Directions

6. **Prospective Research Directions**

Based upon the research in this study, the following future investigations are suggested:

6.1. Future studies integrating basic and applied research that involve *n*-3 and *n*-6 PUFA enriched diets using the same and different basal diets, different levels of *n*-3 and *n*-6 PUFA in diet, and focus on activity and expression of key lipogenic enzymes in muscle and their relationship with rumen metabolism, might help to differentiate lipid metabolism of these kinds of PUFA in bovine muscle and contribute to the development of beef quality. Likewise, it could clarify the increase of *n*-3 PUFA, the decrease of SFA, and the variable changes in VA and CLA observed in the present study in muscle of animals fed *n*-3 PUFA enriched diets.

6.2. New studies to find ways to regulate the intramuscular fat deposition in bovines are necessary.

6.3. Investigations of specific markers of lipid peroxidation should be evaluated in beef muscle.

6.4. Other antioxidants such as ascorbic acid, carnosine, and antioxidant enzymes should be included in next studies that involve antioxidant status of bovine muscle.

6.5. Separate studies on the relationship between fatty acid composition, lipid peroxidation, and antioxidant status of stored muscle should be carried out in order to contribute to the future development of meat animals for consumers.

6.6. Studies of feeding restriction under compensatory and no compensatory growth conditions and its interaction with *n*-3 PUFA enriched diets should involve detailed evaluation of rumen metabolism, expression and activity of muscle lipogenic enzymes in order to verify if feeding restriction alone or plus PUFA enriched diets could really lead to an accumulation of some unsaturated FA such as VA and CLA in bovine muscle. Likewise, studies are necessary to evaluate proteolysis during and after feeding restriction, and during different times of the tenderization process of muscle, relating it to lipid peroxidation, antioxidant status, and tenderness, in order to understand the decrease of tenderness in restricted animals, and to corroborate the hypothesis about the effect of feeding restriction on calpain/calpastatin system.

Summary

It has been demonstrated that fat quality and total fat quantity play an essential role in determining health problems. Fat quality has been related specially with the presence of bioactive FA having important function for human health. It has stimulated the consumer's interest on the fatty acid composition of different animal fat depots and products.

Two different experiments were carried out in order to investigate the influence of feeding *n*-3 and *n*-6 PUFA enriched diets on the relationship of lipid peroxidation and antioxidant status including fatty acid composition and quality in *longissimus* muscle from bulls. The **Experiment 1** was carried out with **Simmental German bulls** under indoor conditions. Control animals were daily fed maize silage/grass silage (70/30, *ad libitum*), molasses, hay and concentrate including soybean. Treatment group I, were fed under unrestricted conditions grass silage, molasses, hay, and concentrate including rapeseed in the same proportion as the control group. Treatment group II fed as treatment group I with a restriction of 1 kg of concentrate (50%) per day. The **Experiment 2** was carried out with **German Holstein bulls.** Control animals fed maize silage, and concentrate enriched with *n*-6 FA (soybean-based concentrate); and treatment animals fed grass silage, and concentrate enriched with *n*-3 FA (linseed oil and rapeseed cake based concentrate).

In the experiment 1, the treatment diet was successful in improving beef fatty acid composition without affecting tenderness and color (under unrestricted conditions). There were no differences in vitamins and cooking loss, but selenium decreased in treatment groups. Stimulated lipid peroxidation, in samples taken immediately post-mortem, was higher in treatment groups. PUFA decreased, SFA, and intramuscular fat increased after 14 days of storage while vitamins had no significant reduction. Catalase and superoxide dismutase activities were

significantly higher in the treatment diet groups, and glutathione peroxidase activity was not different. AOC was not affected by the diet. However, storage affected the values of FRAP and TEAC assays, and the results were time-depending. 30 minutes were found like a minimum reaction time for both assays. Generally, AOC values of the hydrophilic antioxidants were significantly higher than lipophilic values.

In the experiment 2, 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. These 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. Results from both experiments indicates that different treatment *n*-3 PUFA enriched diets improved the fatty acid composition of beef in reference to an increase of beneficial fatty acid content in beef related to human health, especially *n*-3 FA, even under stored conditions. Most of muscle characteristics were not affected by diet under unrestricted conditions. However, *n*-3 PUFA enriched diets from both experiments developed higher lipid peroxidation in muscle compared to *n*-6 PUFA enriched diets. Nevertheless, results were different according to the antioxidant status offered by each diet. Increasing *n*-3 PUFA in beef cattle diets should ensure a correct balance of trace elements, lipid-soluble vitamins, and other antioxidants in order to allow a positive answer in the defense of the antioxidant system against lipid peroxidation.

Zusammenfassung

Zur Bewertung des Nährwertes ist sowohl die qualitative als auch die quantitative Zusammensetzung des Fettes im Fleisch von Bedeutung. Ausgewählter Fettsäuren fungieren als bioaktive Substanzen bei einer Reihe von Prozessen im menschlichen Körper. Seit einigen Jahren bestimmt das Wissen um die Bedeutung z.B. von *n*-3 Fettsäuren das Kaufverhalten der Verbraucher mit.

In zwei großen Studien wurden der Einfluss von *n*-3 und *n*-6 Fettsäure angereichtertem Futter auf die Beziehung zwischen Lipidperoxidation und antioxidativer Kapazität sowie dem Fettsäureprofil im Muskel von Bullen untersucht. Das **Experiment 1** wurde mit **Bullen der Rasse Deutsche Simmentaler** durchgeführt. Nach dem Zufallprinzip wurden 25 Bullen in drei Gruppen zusammengestellt. Die Tiere der Kontrollgruppe wurden mit einer Mischung von Mais- und Grassilage, Molasse, Heu und einem sojahaltigen Konzentrat gefüttert. Die Experimentgruppe I wurde mit Grassilage, Molasse, Heu und einem leinsamenhaltigen Konzentrat gefüttert. Die Experimentgruppe II wurde vergleichbar zur Experimentgruppe I gefüttert, aber mit einer Restriktion. Das **Experiment 2** wurde mit Bullen der **Rasse Deutsche Holsteiner** durchgeführt. Nach dem Zufallprinzip wurden 29 Bullen in zwei Gruppen zusammengestellt. Die Bullen der Kontrollgruppe wurden mit Maissilage und einem sojahaltigen Konzentrat gefüttert. Die Bullen der Experimentgruppe erhielten Grassilage und eine Rapskuchen- und Leinsamenölhaltiges Konzentrat.

Im Ergebnis der Untersuchungen des **Experimentes 1** gelang es die Fettsäurezusammensetzung zu verändern, ohne die Qualität des Muskels (Farbe, Zartheit) zu beeinflussen. Die Fütterung mit Grassilage hatte eine Erhöhung der *n*-3 Fettsäuregehalte und eine Reduzierung der Gehalte ausgewählter gesättigter Fettsäuren im Muskel der Bullen zur Folge. Die Konzentrationen der lipidlöslichen Vitamine wurde nicht durch die unterschiedliche Fütterung beeinflusst. In den

Experimentgruppen waren die Selengehalte signifikant verringert. In den Muskelproben der Experimentgruppen trat eine höhere Lipidperoxidation auf im Vergleich zur Kontrollgruppe. Sowohl die mehrfach ungesättigten Fettsäuren (PUFA), die gesättigten Fettsäuren und der intramuskuläre Fettgehalt wurden signifikant durch dien Prozess der Fleischreifung (14 Tage Lagerung) der Muskelproben erhöht. Die Aktivität der endogenen Enzyme Katalase und Superoxiddismutase war in den Muskelproben der Experimentgruppen höher im Vergleich zur Kontrollgruppe, hingegen zeigte die Fütterung keinen Einffluss auf das Enzym Glutathioperoxidase. Die antioxidative Kapazität in den Muskelproben wurde durch die Fütterung nicht beeinflusst. Im Ergebnis der Untersuchungen stellte sich heraus, dass für die Assays zur Bestimmung der antioxidativen Kapazität im Muskel Mindestreaktionszeit von 30 Minuten erforderlich ist. Die antioxidative Kapazität der hydrophilen Antioxidantien waren bei beiden verwendeten Assays signifikant höher im Vergleich zur Aktivität der lipophilen Antioxidantien.

Im **Experiment 2** führte die unterschiedliche Fütterung ebenfalls zu Veränderungen in der Fettsäurezusammensetzung des Muskels und damit verbunden zur Anreicherung der ernährungsphysiologisch wertvollen *n*-3 Fettsäuren. Desweiteren wurde der Gehalt an ß-Carotin und die Aktivität der Katalase im Muskel der mit Grassilage gefütterten Bullen signifikant gegenüber der Kontrollgruppe erhöht. Auch in Experiment 2 war die Lipidperoxidation im Muskel der Experimentgruppe höher im Vergleich zur Kontrollgruppe. Die antioxidative Kapazität im Muskel der Bullen wurde nicht von der Fütterung beeinflusst.

Die Ergebnisse beider Experimente mit Bullen unterschiedlicher Rasse zeigten, dass ein mit *n*-3 Fettsäuren angereichertes Futter das Fettsäureprofil im Muskel verändert und den Anteil an ernährungsphysiologisch wertvollen längerkettigen *n*-3 Fettsäuren erhöht. Diese trifft auch auf das Fettsäureprofil nach der Fleischreifung (14 Tage Lagerung) zu. Auch eine Futterrestriktion führt nicht zu signifikanten

Veränderungen in den Muskelcharakteristika. Jedoch führt diese mit *n*-3 Fettsäuren angereicherte Futterung bei beiden Experimenten zu einer erhöhten Lipidperoxidation im Vergleich zu den Kontrollgruppen. Dagegen blieb die antioxidative Kapazität im Muskel vom Futter unbeeinflusst. Jedoch sollte bei Fütterung von Grassilage mit *n*-3 fettsäure haltigen Supplementen der Schutz der im Muskel eingelagerten PUFA gegenüber Lipidperoxidation durch Einsatz geeigneter Antioxidantien berücksichtigt werden.

References

Abbas, K.A., Mohamed, A., & Jamilah, B. (2009). Fatty acids in fish and beef and their nutritional values: A review. *Journal of Food, Agriculture & Environment*, *7* (3-4), 37-42.

Abegaz, S., Tiyo, D., & Gizachew, L. (1996). Compensatory growth in horro lambs of Ethiopia. IN: Small ruminant Research and Development in Africa. Edited by Lebbie, S.H.B., & Kagwini, E. Proceedings of the Third Biennial Conference of the African Small Ruminant Research Network. UICC, Kampala, Uganda, 5-9 December 1994. ILRI (International Livestock Institute) Nairobi, Kenya. p 209-213.

Aebi, H.E. (1974). Catalase. *Methods for enzymatic analysis. Vol. 1* (pp. 273-286). Verlag Chemie, Weinheim: HU. Bergmeyer ed.

Almendingen, K., Jordal, O., Kierulf, P., Sandstad, B., & Pedersen, J.I. (1995). Effects of partially hydrogenated fish oil, partially hydrogenated soybean oil, and butter on serum lipoproteins and Lp[a] in men. *Journal of Lipid Research*, *36*, 1370-1384.

Arnold, R.N., Arp, S.C., Scheller, K.K., Williams, S.N., & Schaefer, D.M. (1993). Tissue equilibrium and subcellular distribution of vitamin E relative to myoglobin and lipid oxidation in displayed beef. *Journal of Animal Science,71*, 105-118.

Barciela, J., Herrero, C., García-Martín, S., & Peña, R.M. (2008). A brief study of the role of selenium as antioxidant. *Electronic Journal of Environmental, Agricultural, and Food Chemistry, 7 (8),* 3151-3155.

Bassett, Ch.M.C, Edel, A.L., Patenaude, A.F., McCullough, R.S., Blackwood, D.P., Chouinard, P. Y., Paquin, P., Lamarche, B., & Pierce, G.N. (2010). Dietary Vaccenic Acid Has Antiatherogenic Effects in LDLr–/– Mice. *The Journal of Nutrition*,*140*, 18-24.

Bauman, D. E., Perfield, J.W., de Veth, M.J., & Lock, A.L. (2003). New perspectives on lipid digestion and metabolism in ruminants. *Proc. Cornell Nutr. Conf*, 175-189.

Bauman, D. E., Baumgard, l.h., Corl, b.a., & Griinari, M. (1999). Biosynthesis of conjugated linoleic acid in ruminants in *Proc. Am. Soc. Anim. Sci*. http://jas.fass.org/misc/abstr_proc.shtml Accessed May 14, 2010.

Bauchart, D., Gobert, M., Habeanu, M., Parafita, E., Gruffat, D., & Durand, D. (2009). Effects of dietary *n*-3 polyunsaturated fatty acids and antioxidants on beef fatty acids and lipoperoxidation in meat-producing cattle. 1st Workshop XIII International Feed Technology Symposium in Serbian from September 29th-October 1st.

Bauchart, C., Rémond, D., Chambon, C., Patureau Mirand, P., Savary-Auzeloux, I., Reynés, C., & Morzel, M. (2006). Small peptides (<5 kDa) found in ready-to-eat beef meat. *Meat Science, 74,* 658–666.

Benzie, I.F.F., & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP Assay. *Analytical Biochemistry*, *239*, 70-76.

Brouwer, I.A., Wanders, A.J., & Katan, M.B. (2010). Effect of Animal and Industrial *Trans* Fatty Acids on HDL and LDL Cholesterol Levels in Humans – A Quantitative Review. *Plos one*, *5* (3),1-10.

Bergen, W.G., & Mersmann, H.J. (2005). Comparative Aspects of Lipid Metabolism: Impact on Contemporary Research and Use of Animal Models. *The Journal of Nutrition, 135,* 2499-2502.

Bobček, B., Lahučký, R., Mrázová, J., Bobček, R., Novotná, K., & Vašíček, D. (2004). Effects of dietary organic selenium supplementation on selenium content, antioxidative status of muscles and meat quality of pigs. *Czech Journal of Animal Science, 49, 411–417.*

Calder, P.C. (2008). Polyunsaturated fatty acids, inflammatory process and inflammatory bowel diseases (review). *Molecular Nutrition & Food Research*, *8*, 885-897.

Cabaraux, J.F., Dotreppe, O., Dufrasne, I., Clinquart, A., Istasse, L., & Hornick, J.L. (2004). Effect of reducing energy supply during the finishing of Belgian Blue double-muscled cull cows. *Animal Science*, *79*, 469-482.

Cha, M.C., & Jones, P.J.H. (1996). Tissue fatty acid deposition is influenced by an interaction of dietary oil source and energy intake level in rats. *Journal of. Nutritional Biochemistry*, *7*, 650-658.

Chartrin, P., Méteau, K., Juin, H., Bernadet, M.D., Guy, G., Larzul, C., Rémignon, H., Mourot, J., Duclos, M.J., & Baéza, E. (2006). Effects of Intramuscular Fat Levels on Sensory Characteristics of Duck Breast Meat. *Poultry Science, 85*, 914–922.

Chen, Z.Y., & Cunnane, S.C. (1992). Preferential retention of linoleic acid enriched triacylglycerols in liver and serum during fasting. *American Journal of Physiology*, *263*, R233-R239.

Cho, E.S., Sahyoun, N., & Stegink, L.D. (1981). Tissue glutathione as a cysteine reservoir during fasting and refeeding of rats*. Journal of Nutrition, 111,* 914-922.

Cerdeño, A., Vieira, C., Serrano, E., Lavın, P., & Mantecón, A. R. (2006). Effects of feeding strategy during a short finishing period on performance, carcass and meat quality in previously-grazed young bulls. *Meat Science, 72*, 719-726.

Chang, J.P-Ch., Yi-Ting Chen, Y-T., & Su, K-P. (2009). Omega-3 Polyunsaturated Fatty Acids (*n*-3 PUFAs) in Cardiovascular Diseases (CVDs) and Depression:The Missing Link? *Cardiovascular Psychiatry and Neurology*, *725310*, 1-6.

Chaijan, M. (2008). Review: Lipid and myoglobin oxidations in muscle foods. *Songklanakarin Journal of Science and Technology*, *30* (1), 47-53.

Chen, Y.J., Zhou, G. H., Zhu, X.D., Xu, X.L., Tang, X.Y., & Gao, F. (2007). Effect of low dose gamma irradiation on beef quality and fatty acid composition of beef intramuscular lipid. *Meat Science, 75*, 423-431.

Corino, C., Magni, S., Pagliarini, E., Rossi, R., Pastorelli, G., & Chiesa, L.M. (2002). Effects of dietary fats on meat quality and sensory characteristics of heavy pig loins. *Meat Science, 60,* 1-8.

Crehan, C.M., E. Hughes, D.J. Troy., & Buckley, D.J. (2000). Effects of fat level and maltodextrin on the functional properties of frankfurters formulated with 5, 12 and 30% fat. *Meat Science*, *55*, 463-469.

Daley, C.A., Abbott, A., Doyle, P.S., Nader, G.A., & Larson, S. (2010). A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutrition Journal, 9* (10), 1-12.

Daly, C.M., Moloney, A.P., & Monahan, F.J. (2007). Lipid and colour stability of beef from grazing heifers supplemented with sunflower oil alone or with fish oil *Meat Science*, 77 (4), 634-642.

Dannenberger, D., Nuernberg, K., Nuernberg, G., & Ender, K. (2006). Carcass-and meat quality of pasture vs concentrate fed German Simmental and German Holstein bulls. *Archiv für Tierzucht (Archives of Animal Breeding), 4*, 315-328.

Dannenberger, D., Reichardt, W., Danier, J., Nürnberg, K., Nürnberg, G., & Ender, K. (2007). Aktuelle Untersuchungen zu Gehalten ausgewählter Spurenelemente im Fleisch deutscher Schweinerassen. *Fleischwirtschaft, 87* (6), 90-93.

Dannenberger, D., Reichardt, W., Danier, J., Nuernberg, K., Nuernberg, G., & Ender, K. (2007). Investigations on selected essential micronutrients in muscle of German pure and crossbred pigs. *Fleischwirtschaft, 87* (6), 90-93.

Deckelbaum, R.J., & Calder, Ph. C. (2010). Dietary *n*-3 and *n*-6 fatty acids: are there 'bad' polyunsaturated fatty acids? *Current Opinion in Clinical Nutrition and Metabolic Care*, *13*,123-124.

Del Campo, M., Brito, G., Soares de Lima, J.M., Vaz Martins, D., Sañudo, C., San Julián, R., Hernández, P., & Montossi, F. (2008). Effects of feeding strategies including different proportion of pasture and concentrate, on carcass and meat quality traits in Uruguayan steers. *Meat Science, 80*, 753–760.

De La Torre, A., Gruffat, D., Durand, D., Micol, D., Peyron, A., Scislowski, V., & Bauchart, D. (2006). Factors influencing proportion and composition of CLA in beef. *Meat Science, 73*, 258-268.

Descalzo, A.M., Rossetti, L., Sancho, A.M., Garcia, P.T., Biolatto, A., Carduza, F., & Griogioni, G. M. (2008). Antioxidant consumption and development of oxidation during ageing of buffalo meat produce in Argentina. *Meat Science*, *79*, 582–588.

Descalzo, A.M., & Sancho, A.M. (2008). A review of natural antioxidants and their effects on oxidative status, odour and quality of fresh beef produced in Argentina. *Meat Science, 79*, 423-436.

Descalzo, A. M., Rossetti, L., Grigioni, G., Irurueta, M., Sancho, A. M., Carrete, J., & Pensel, N.A. (2007). Antioxidant status and odour profile in fresh beef from pasture or grain-fed cattle. *Meat Science*, *75*, 299-307.

Descalzo, A.M., Insani, E.M., Biolatto, A., Sancho, A.M., Garcıa, P.T., Pensel, N.A., & Josifovich, J.A. (2005). Influence of pasture or grain-based diets supplemented with vitamin E on antioxidant/oxidative balance of Argentine beef. *Meat Science*, *70*, 35-44.

DeVore, V.R., Colnago, G.L., Jensen, L.S., & Greene, B.E. (1983). Thiobarbituric Acid Values and Glutathione Perioxidase Activity in Meat from Chickens Fed a Selenium-Supplemented Diet. *Journal of Food Science*, *48* (1), 300-306.

Dhiman, T.R., Nam, S.H., & Ure, A.L. (2005). Factors affecting conjugated linoleic acid content in milk and meat. *Critical Reviews in Food Science and Nutrition*, *45* (6), 463-82.

De Smet, S., Raes, K., & Demeyer, D. (2004). Meat fatty acid composition as affected by fatness and genetic factors: A review. *Animal Research, 53,* 81-98.

EFSA (European Food Safety Authority). (2010). Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. The *EFSA Journal, 8*(3), 1461-1568.

EFSA (European Food Safety Authority), 2009. Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies on a request from Mead Johnson Nutritionals on DHA and ARA and visual development. *The EFSA Journal, 94*, 1-14.

Enser, M., Scollan, N.D., Chi, J.J., Kurt, E., Hallett, K., & Wood, J.D. (1999). Effect of dietary lipid on the content of conjugated linoleic acid (CLA) in beef musle. *Animal Science*, *69*, 143-146.

Facco, E., Lage, M., & Godoy, H. (2009). Influence of vitamin E supplemented diet on charque quality and lipid stabilization. *Brazilian archives of biology and technology*, *52* (3), 729-736.

Field, C.J., Blewett, H.H., Proctor, S., & Vine, D. (2009). Human health benefits of vaccenic acid. *Applied Physiology, Nutrition and Metabolism*, *34*, 979-991.

Filaire, E., Rouveix, M., Massart, A., Gladine, C., Davicco, M.J, & Durand, D. (2009). Lipid peroxidation and antioxidant status in rat: effect of food restriction and wheel running. *European Journal of Applied Physiology*,*107*(2), 243-50.

Folch, J., Lees, G.H., & Sloane-Stanley. (1956). A simple method for the isolation and purification of total lipids from animal tissue. *Journal of Biological Chemistry, 226*, 497-509.

Fredriksson, S & Pickova, J. (2007). Fatty acids and tocopherol levels in M. *Longissimus dorsi* of beef cattle in Sweden. A comparison between seasonal diets. *Meat Science, 76*, 746-754.

French, P., Stanton, C., Lawless, F., O'Riordan, E.G., Monahan, F.J., Caffrey, P.J., & Moloney, A.P. (2000). Fatty acid composition, including conjugated linoleic acid,

of intramuscular fat from steers offered grazed grass, grass silage, or concentrate-based diets. *Journal of Animal Science*, *78*, 2849-2855.

Fyfe, D. (2000). Determination of antioxidant capacity in human serum using the Cary Eclipse for the ORAC assay. Varian Australia Pty Ltd, Mulgrave, Victoria 3170, Australia.

Garassino, M., Montorfano, G., Fallini, M., Adorni, M., Berra, B., & Rizzo, A. (2006). A new biomarker in cancer patients: The arachidonic acid/eicosapentaenoic acid (AA/EPA) ratio First AACR International Conference on Molecular Diagnostics in Cancer Therapeutic Development, Sep 12-15.

Gatellier, P., Mercier, Y., Juin, H., & Renerre, M. (2005). Effect of finishing mode (pasture- or mixed-diet) on lipid composition, colour stability and lipid oxidation in meat from Charolais cattle. *Meat Science*, *69*, 175-186.

Gatellier, P., Mercier, Y., & Renerre, M. (2004). Effect of diet finishing mode (pasture or mixed diet) on antioxidant status of Charolais bovine meat. *Meat Science, 67,* 385-394.

Gatellier, P., Hamelin, C., Durand, Y., & Renerre, M. (2001). Effect of a dietary vitamin E supplementation on color stability and lipid oxidation of air- and modified atmosphere-packaged beef. *Meat Science, 59*, 133-140.

Gebicki, S., & Gebicki, J. M. (1993). Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. *Biochemical Journal*, *289,* 743-749.

Ghiselli, A., Serafini, M., Natella, F., & Scaccini, Cr. (2000). Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radical Biology & Medicine*, *29* (11), 1106-1114.

Goicoechea, S.M., & Conde, R.D. (1997). Influence of protein nutrition on calpain activity of mouse kidney: Modulation by calpastatin. *Molecular and Cellular Biochemistry*, *166*, 95-99.

González, L., Bispo, E., Moreno, T., Monserrat, L., & Franco, D. (2008). Enhancement of shelf life from meat proceeding from Blond Galician calves using antioxidant. *EJEAFChe, 7,* 3296-3300.

Gopalakrishnan, J., Decker, E.A., & Means, W.J. (1999). Antioxidant activity of mechanically separated pork extracts. *Meat Science, 52,* 101-110.

Griinari, J.M., & Bauman, D.E. (1999). Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk of ruminants. In: *Advances in Conjugated Linoleic Acid Research*, Volume I. Eds. Yurawecz, M.P., Mossoba, M.M., Kramer, J.K.G., Pariza, MW., Nelson, G.J. AOCS press, Illinois, Chapter 13, 180-200.

Gupta, R., Sharma, M., Lakshmy, R., Prabhakaran, D., & Reddy, K.S. (2009). Improved method of total antioxidant assay. *Indian Journal of Biochemestry & Biophysics*, *46* (1), 126-129.

Guttmann, R.P., & Johnson, G.V.W. (1998). Oxidative Stress Inhibits Calpain Activity in Situ. *The Journal of Biological Chemistry*, *273* (21), 13331-1338.

Hansen, S., Therkildsen, M., & Byrne, D.V. (2006). Effects of a compensatory growth strategy on sensory and physical properties of meat from young bulls. *Meat Science*, *74* (4), 628-643.

Harfoot, CG & Hazlewood, G.P. (1997). The Rumen Microbial Ecosystem. In: *Lipid metabolism in the rumen*. PN. Hobson, SC. Stewart (ed), Blackie Academic & Professional, London, UK, 382-426.

Herdmann, A., Nuernberg, K., Martin, J., Nuernberg, G., & Doran, O. (2010a). Effect of dietary fatty acids on expression of lipogenic enzymes and fatty acid profile in tissues of Bulls. *Animal*, *4*, 755-762.

Herdmann, A., Martin, J., Nuernberg, G., Dannenberger, D., & Nuernberg, K. (2010b). How does *n*-6 and *n*-3 PUFA enriched diet affects the fatty acid composition of different tissues of German Simmental bulls? *Meat Science.* doi: 10.1016/j.meatsci.2010.06.004.

Herdmann, A., Martin, J., Nuernberg, G., Dannenberger, D., & Nuernberg, K. (2010c). Transfer of dietary *n*-3 and *n*-6 PUFA into different tissues of German Holstein bulls and the fate of bioactive fatty acids during processing. *Journal of Agricultural and Food Chemistry*. Revision submitted.

Hernandez, P., Zomenno, L., Arinno, B., & Blasco, A. (2004). Antioxidant, lipolytic and proteolysis enzyme activities in pork meat from different genotypes. *Meat Science*, 66, 525-529.

Hocquette, J.F., Cassar-Malek, I., Bernard-Capel, C., & Picard, B. (2009). Functional genomics and new markers for beef production – mini-review. *Animal Science Papers and Reports*, 27 (4), 273-280.

Hornick, J.L., Van Eenaeme, C., Clinquart, A., Diez, M., & Istasse, L. (1998). Different periods of feed restriction before compensatory growth in Belgian Blue bulls: I. animal performance, nitrogen balance, meat characteristics, and fat composition. *Journal of Animal Science, 76*, 249-259.

Huang, D., Ou, B., & Prior, R.L. (2005). The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry, 53,* 1841-1856.

Hynes, G.R., Heshka, J., Chadee, K., & Jones, P.J. (2003). Effects of dietary fat type and energy restriction on adipose tissue fatty acid composition and leptin production in rats. *Journal of Lipid Research*, *44*(5), 893-901.

Ikeda, I., Cha, J.Y., Yanagita, T., & Nakatani, N. (1998). Effects of dietary α-linolenic, eicosapentaenoic and docosahexaenoic acids on hepatic lipogenesis and β-oxidation in rats. *Bioscience, Biotechnology, and Biochemistry Journal*, *62*, 675-680.

Insani, E.M., Eyherabide, A., Grigioni, G., Sancho, A.M., Pensel, N.A., & Descalzo, A.M. (2008). Oxidative stability and its relationship with natural antioxidants during refrigerated retail display of beef produced in Argentina. *Meat Science, 79,* 444-452.

Jakobsen, M.U., Overvad, K., Dyerberg, J., & Heitmann, B.L. (2008). Intake of ruminant *trans* fatty acids and risk of coronary heart disease. *International Journal of Epidemiology*, *37*, 173-182.

Jaramillo, S., Villa, N.A., Pineda, A.F., Gallego, A.B., Tabares, P., & Ceballos, A. (2005). Actividad sanguínea de superóxido dismutasa y glutatión peroxidasa en novillas a pastoreo. *Pesquisa Agropecuária Brasileira, 40 (11) ,*1115-1121.

Joseph, S.J., Robbins, K.R., Pavan, E., Pratt, S.L., Duckett, S.K., & Rekaya, R. (2010). Effect of diet supplementation on the expression of bovine genes associated with Fatty Acid synthesis and metabolism. *Bioinformatics and Biology Insights, 4*, 19-31.

Jouany, J.B., Lassalas, B., Doreau, M., & Glasser, F. (2007). Dynamic features of the rumen metabolism of linoleic, linolenic acid and linseed oil measured *in vitro*. *Lipids*, *42*, 351-360.

Karunasinghe, N., Ferguson, L.R., Tuckey, Jh., & Masters, Jh. (2006). Hemolysate Thioredoxin Reductase and Glutathione Peroxidase Activities Correlate with Serum Selenium in a Group of New Zealand Men at High Prostate Cancer Risk. *The Journal of Nutrition*, *136*, 2232-2235.

Keady, T.W.J., Lively, F.O., Kilpatrick, D.J., & Moss, B.W. (2007). Effects of replacing grass silage with either maize or whole-crop wheat silages on the performance and meat quality of beef cattle offered two levels of concentrates. *Animal, 1,* 613-623.

Kelly, N.S., Hubbard, N.E., & Erickson, K.L. (2007). Conjugated linoleic isomers and cancer. *Journal of Nutrition, 137*, 2599-2607.

Kenny, D.A., Kelly, J.P., Monahan, F.J., & Moloney, A.P. (2007). Effect of dietary fish and soya oil on muscle fatty acid concentrations and oxidative lipid stability in beef cattle. *Journal of Animal Science*, *85* (1), 911.

Kim, S.C., Adesogan, A.T., Badinga, L., & Staples, C.R. (2007). Effects of dietary *n*-6/*n*-3 fatty acid ratio on feed intake, digestibility, and fatty acid profiles of the ruminal contents, liver, and muscle of growing lambs. *Journal of Animal Science, 85* (3), 706-16.

Kim, H., Choi, S., Lee, H.J., Lee, J.H., & Choi, H. (2003). Suppression of fatty acid synthase by dietary polyunsaturated fatty acids is mediated by fat itself, not by peroxidative mechanism. *Journal of Biochemistry and Molecular Biology*, *36* (3), 258-264.

Kohjima, M., Enjoji, M., Higuchi, N., Kato, M., Kotoh, K., Nakashima, M., & Nakamuta, M. (2009). The effects of unsaturated fatty acids on lipid metabolism in HepG2 cells. *In Vitro Cellular & Developmental Biology – Animal*, *45*, 6-9.

Koohmaraie, M., & Geesink, G.H. (2006). Contribution of post-mortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Science*, *74*, 34-43.

Koutsidis, G., Elmore, J.S., Oruna-Concha, M.J., Campo, M.M., Wood, J.D., & Mottram, D.S. (2008). Water-soluble precursors of beef flavour. Part II: Effect of post-mortem conditioning. *Meat Science, 79,* 270-277.

Kris-Etherton, P.M., & Yu, S. (1997). Individual fatty acid effects on plasma lipids and lipoproteins: human studies. *American Journal of Clinical Nutrition, 65*, 1628S-1644S.

Kuechenmeister, U., Nuernberg, K., Fiedler, I., Kuhn, G., Nuernberg, G., & Ender, K. (1999). Cell injury and meat quality of pig in the time period post-mortem from two genotypes susceptible or resistant to malignant hyperthermia. *European Food Research and Technology*, *206*, 97–103.

Lazarus, C. R., Deng, J. C., & Watson, C. M. (1977). Changes in the concentrations of fatty acids from the no polar, phospho- and glycolipids during storage of intact lamb muscle. *Journal of Food Science, 42*, 102–107.

Lehnert, S.A., Byrne, K.A., Reverter, A., Nattrass, G.S., Greenwood, P.L., Wang, YH., Hudson, N.J., & Harper, G.S. (2006). Gene expression profiling of bovine skeletal muscle in response to and during recovery from chronic and severe under-nutrition. *Journal of Animal Science*, *84*, 3239-3250.

Linden, S. (2003). α-Tocoperol, β-Carotin und L-Ascorbinsäure in der Rindermast – Einfluss auf Qualitätsparameter. Dissertation Universität Hamburg. 242 p.

Loerch, S.C. (1990). Effects of feeding growing cattle high-concentrate diets at a restricted intake on feedlot performance. *Journal of Animal Science*, *68*, 3086-3095.

Luhtala, T.A., Roecker, E.B., Pugh, T., Feuers, R.J., & Weindruch, R. (1994). Dietary restriction attenuates age-related increases in rat skeletal muscle antioxidant enzyme activities. *Journal of Gerontology Biological Science, 49*, 231-238.

Łukaszewicz, M., Szopa, J., & Krasowska, A. (2004). Susceptibility of lipids from different flax cultivars to peroxidation and its lowering by added antioxidants. *Food Chemistry*, *88*, 225-231.

Lusk, J.L., & Parker, N. (2009). Consumer Preferences for Amount and Type of Fat in Ground Beef. *Journal of Agricultural and Applied Economics*, *41* (1), 75-90.

Mach, N., Devant, M., Díaz, I., Font-Furnols, M., Oliver, M.A., García, J.A., & Bach, A. (2006). Increasing the amount of *n*-3 fatty acid in meat from young Holstein bulls through nutrition. *Journal of Animal Science*, *84*, 3039-3048.

McDonagh, M.B., Fernandez, C., & Oddy, V.H. (1999). Hind-limb protein metabolism and calpain system activity influence post-mortem change in meat quality in lamb. *Meat Science*, *52*, 9-18.

Mader, T.L., Holt, S.M., Hahn, G.L., Davis, M.S., & Spiers, D.E. (2002). Feeding strategies for managing heat load in feedlot cattle. J*ournal of Animal Science*, *80* (9), 2373-82.

Mahecha, L., Nuernberg, K., Nuernberg, G., Ender, K., Hagemann, E., & Dannenberger, D. (2009). Effects of diet and storage on fatty acid profile,

micronutrients and quality of muscle from German Simmental bulls. *Meat Science*, *82*, 365–371.

Mahecha, L., Nuernberg, K., Nuernberg, G., Martin, J., Hubbermann, E.M., Knoeller, S., Claeys, E., De Smet, S., & Dannenberger, D. (2010a). Antioxidant enzyme activities and antioxidant capacity in beef muscle from bulls fed diets rich in polyunsaturated fatty acids. *Food Chem*istry. Revision submitted.

Mahecha, L., Dannenberger, D., Nuernberg, K., Nuernberg, G., Hagemann, E., & Martin, J. (2010 b). Relationship between Lipid Peroxidation and Antioxidant Status in the Muscle of German Holstein Bulls Fed *n*-3 and *n*-6 PUFA-Enriched Diets. *Journal of Agricultural and Food Chemistry*. DOI:10.1021/jf101218b

Maltin, Ch., Balcerzak, D., Tilley, R., & Delday, M. (2003). Determinants of meat quality: tenderness. *Proceedings of the Nutrition Society, 62*, 337–347.

Mashima, T., Seimiya, H., & Tsuruo, T. (2009). *De novo* fatty-acid synthesis and related pathways as molecular targets for cancer therapy. *British Journal of Cancer*, *100*, 1369-1372.

Mason, L.M., Hogan, S.A., Lynch, A., O-Sullivan, K., Lawlor, P.G., & Kerry, J.P. (2005). Effects of restricted feeding and antioxidant supplementation on pig performance and quality characteristics of *longissimus dorsi* muscle from Landrace and Duroc pigs. *Meat Science, 70,* 307–317.

Mayne, C.S., & O'Kiely, P. (2005). An overview of silage production and utilisation in Ireland (1950–2005). In: R.S. Park and M.D. Stronge, Editors, *Proceedings of the XIV International Silage Conference, July 2005*, Wageningen Academic Publishers, Belfast, N. Ireland (2005), pp. 19-34.

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of biochemistry, 47,* 469-474.

Mercier, Y., Gatellier, O., & Renerre, M. (2004). Lipid and protein oxidation *in vitro*, and antioxidant potential in meat from Charolais cows finished on pasture or mixed diet. *Meat Science, 66,* 467-473.

Mete, N., Isik, B., Erdinç, L., & Gürkan, F. (1999). The Effect of Fish Oil on Liver and Plasma MDA and Antioxidant Status of Rats. The Effect of Fish Oil on Liver and Plasma MDA and Antioxidant Status of Rats. *Turkish Journal of Medical Sciences, 29,* 1-6.

Migdał, W., Živković, B., & Migdał, L. (2009). Health promoting values of beef. *Biotechnology in Animal Husbandry*, *25* (5-6), 591-596.

Miller, J.R., Siripurkpong, P., Hawes, J., Majdalawieh, A., Ro, H-S., & McLeod, R.S. (2008). The *trans*-10,*cis*-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPARg-dependent and PPARg-independent mechanisms. *Journal of Lipid Research*, *49*, 550-562.

Miller, A., McHarth, E., Stanton, C., & Devery, R. (2003). Vaccenic acid (t11 C18:1) is converted to c9t11 CLA in MCF-7 and SW480 cancer cells. *Lipids*, *38*, 623-632.

Min, B., Nam, K.C., Cordray, J., & Ahn, D.U. (2008). Endogenous factors affecting oxidative stability of beef loin, pork loin, and chicken breast and thigh meats. *Journal of Food Science, 73(6),* 439-446.

Mir, P.S., McAllister, T.A., Scott, S., Aalhus, J., Baron, V., McCartney, D., Charmley, E., Goonewardene, L., Basarab, J., Okine, E., Weselake, R.J., & Mir, Z.

(2004). Conjugated linoleic acid–enriched beef production. *Clin Nutr*, *79*(suppl), 1207S–11S.

Mir, P.S., McAllister, T.A., Zaman, S., Jones, M.S.D., He, M.L., Aalhus, J.L., Jeremiah, L.E., Goonewardene, L.A., Weselake, R.J., & Mir, Z. (2003). Effect of dietary sunflower oil and vitamin E on beef cattle performance, carcass characteristics and meat quality. *Canadian Journal of Animal Science*, *83*, 53-66.

Moibi, J.A., Ekpe, E.D., & Christopherson, R.J. (2000). Acetyl-CoA carboxylase and fatty acid synthase activity and immunodetectable protein in adipose tissues of ruminants: effect of temperature and feeding level. *Journal of Animal Science*, *78*, 2383-2392.

Moloney, A.P., Scollan, N.D., & Miles, L. (2008). Enrichment of *n*-3 fatty acids and conjugated linoleic acid in beef: ProSafeBeef. *Trition Bulletin*, *33* (4), 374-381.

Morimoto, K.C., Van Eenennaam, A.L., DePeters, E.J., & Medrano, J.F. (2005). Hot Topic: Endogenous Production of *n*-3 and *n*-6 Fatty Acids in Mammalian Cells. *Journal of Dairy Science*, *88*, 1142–1146.

Mozaffarian, D. (2008). Fish and *n*-3 fatty acids fort he prevention of fatal coronary heart disease and sudden cardiac death. *American Journal of Clinical Nutrition, 87*, 1991-1996.

Muir, P.D., Smith, N.B., Dobbie, P.M., Smith, D.R., & Bown, M.D. (2001). Effects of growth pathway on beef quality in 18-month-old Angus and South Devon Angus pasture-fed steers. *Animal Science*, *72*, 297-308.

Müller-Nordhorn, J., Binting, S., Roll, S., & Willich, S.N. (2008). An update on regional variation in cardiovascular mortality within Europe. *European Heart Journal, 29*(10), 1316-26.

Nagalakshmi, D., Dhanalakshmi, K., & Himabindu, D. (2009). Effect of dose and source of supplemental zinc on immune response and oxidative enzymes in lambs. *Veterinary Research Communications*, 10.1007/s11259-009-9212-9.

Nilsson, A., Åhman, B., Murphy, M., & Soveri, T. (2006). Rumen function in reindeer (*Rangifer tarandus tarandus*) after sub-maintenance feed intake and subsequent feeding. *Rangifer*, *26* (2), 73-83.

Noci, F., Monahan, F.J., Scollan, N.D., & Moloney, A.P. (2007). The fatty acid composition of muscle and adipose tissue of steers offered unwilted or wilted grass silage supplemented with sunflower oil and fish oil. *British Journal of Nutrition*, *97*, 502-513.

Nuernberg, K., Nuernberg, G., & Dannenberger, D. (2009). Nutrient and lipid composition of muscle in wild animals. *Fleischwirtschaft*, *10*, 99-102.

Nuernberg, K., Kuechenmeister, U., Nuernberg, G., Hartung, M., Dannenberger, D., & Ender, K. (2006). Effect of storage and grilling on fatty acids in muscle of pigs fed plant oils. *European Journal of Lipid Science and Technology, 108(7),* 554-560.

Nuernberg, K., Dannenberger, D., Nuernberg, G., Ender, K., Voigt, J., Scollan, N. D., Wood, J.D., Nute, G.R., & Richardson, R.I. (2005). Effect of a grass-based and a concentrate feeding system on meat quality characteristics and fatty acid composition of *longissimus* muscle in different cattle breeds. *Livestock Production Science, 94*(1-2), 137-147.

Nuernberg, K., Nuernberg, G., Ender, K., Lorenz, S., Winkler, K., Rickert, R., & Steinhart, H. (2002). *n*-3 fatty acids and conjugated linoleic acids of *longissimus* muscle in beef cattle. *European Journal of Lipid Science and Technology, 104,* 463-471.

Nuernberg, K, Kuechenmeister, U., Nuernberg, G., Ender, K., & Hackl, W. (1999). Influence of exogenous application of *n*-3 fatty acids on meat quality, lipid composition, and oxidative stability in pigs. *Archives of Animal Nutrition*, *52*, 53-65.
Oka, A., Iwaki, F., Dohgo, T., Ohtagaki, S., Noda, M., Shiozaki, T., Endoh, O., & Ozaki, M. (2002). Genetic effects on fatty acid composition of carcass fat of Japanese Black Wagyu steers. *Journal of Animal Science, 80,* 1005-1011.

O'Sullivan, A., O'Sullivan, K., Galvin, K., Moloney, A.P., Troy, D.J., & Kerry, J.P. (2002). Grass silage *versus* maize silage effects on retail packaged beef quality. *Journal of Animal Science, 80,* 1556-1563.

Paiva, S., & Rusell, R. (1999). Review Series: Antioxidants and their Clinical Applications β-Carotene and Other Carotenoids as Antioxidants. *Journal of the American College of Nutrition*, *18* (5), 426-433.

Paniagua, J.A., de la Sacristana, A.G., Sánchez, E., Romero, I., Vidal-Puig, A., Berral, F.J., Escribano, A., Moyano, M.J., Peréz-Martinez, P., López-Miranda, J., & Pérez-Jiménez, F. (2007). A MUFA-rich diet improves posprandial glucose, lipid and GLP-1 responses in insulin-resistant subjects. *American College of Nutrition*, *26* (5), 434-444.

Paton, C.M., & Ntambi, J.M. (2009). Biochemical and physiological function of stearoyl-CoA desaturase. *American Journal of Physiology, Endocrinology, and Metabolism, 297* (1), E28-37.

Pavan, E., & Duckett, S.K. (2007). Corn oil supplementation to steers grazing endophyte-free tall fescue. II. Effects on *longissimus* muscle and subcutaneous adipose fatty acid composition and stearoyl-CoA desaturase activity and expression. *Journal of Animal Science*, *85,* 1731-1740.

Petron, M.J., Raes, K., Claeys, E., Lourenc¸o, M., Fremaut, D., & De Smet, S. (2007). Effect of grazing pastures of different botanical composition on antioxidant enzyme activities and oxidative stability of lamb meat. *Meat Science, 75,* 737-745.
Pfalzgraf, A., Frigg, M., & Steinharth, H. (1995). α-Tocopherol contents and lipid oxidation in pork muscle and adipose tissue during storage. *Journal of agricultural and food chemistry, 43*(5), 1339-1342.

Pietinen, P., Ascherio, A., Korhonen, P., Hartman, A.M., Willett, W.C., Albanes, D., & Virtamo, J. (1997). Intake of fatty acids and risk of coronary heart disease in a cohort of Finnish men. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *American Journal of Epidemiology*, *145*, 876-887.

Prior, R.L., & Cao, G. (1999). *In vivo* total antioxidant capacity: Comparison of different analytical methods. *Free Radical Biology & Medicine*, *27*, 1173–1181.

Purchas, R.W., Burnham, D.L., & Morris, S.T. (2002). Effects of growth potential and growth path on tenderness of beef *longissimus* muscle from bulls and steers. *Journal of Animal Science*, *80* (12), 3211-3221.

Raes, K., De Smet, S., & Demeyer, D. (2004). Effect of dietary fatty acids on incorporation of long chain polyunsaturated fatty acids and conjugated linoleic acid in lamb, beef and pork meat: a review. *Animal Feed Science and Technology*, *113*, 199-221.

Raes K, De Smet S, Balcaen A, Claeys E, & Demeyer D. (2003). Effect of diets rich in *n*-3 polyunsatured fatty acids on muscle lipids and fatty acids in Belgian Blue double-muscled young bulls. *Reproduction Nutrition Development*, *43*, 331-345.

Ramsden, C.E., Faurot, K.R., Carrera-Bastos, P., Cordain, L., De Lorgeril, M., & Sperling, L.S. (2009). Dietary fat quality and coronary heart disease prevention: a unified theory based on evolutionary, historical, global, and modern perspectives. *Current Treatment Options in Cardiovascular Medicine*, *11*(4), 289-301.

Ratnayake, W.M., & Galli, C. (2009). Fat and Fatty Acid Terminology, Methods of Analysis and Fat Digestion and Metabolism: A Background Review Paper. *Annals of Nutrition and Metabolism*, *55*, 8-43.

Razminowicz, R.H., Kreuzer, M., Leuenberger, H., & Scheeder, M.R.L. (2008). Efficiency of extruded linseed for the finishing of grass-fed steers to counteract a decline of omega-3 fatty acids in the beef. *Livestock Science, 114*, 150-163.

Renerre, M., Poncet, K., Mercier, Y., Gatellier, Ph., & Metro, B. (1999). Influence of Dietary Fat and Vitamin E on Antioxidant Status of Muscles of Turkey. *Journal of Agricultural and Food Chemistry*, *47*, 237-244.

Rhee, K.S., Dutson, T.R., & Smith, G.C. (2006). Enzymic Lipid Peroxidation in Microsomal Fractions from Beef Skeletal Muscle. *Journal of Food Science, 49* (3), 675-679.

Rodríguez-Alcalá, L.M., & Fontecha, J. (2007). Hot Topic: Fatty Acid and Conjugated Linoleic Acid (CLA) Isomer Composition of Commercial CLA-Fortified Dairy Products: Evaluation After Processing and Storage. *Journal of Dairy Science, 90* (5), 2083-2090.

Rohrbach, S., Gruenler, S., Teschner, M., & Holtz, J. (2006). The thioredoxin system in aging muscle: key role of mitochondrial thioredoxin reductase in the protective effects of caloric restriction? *American Journal of Physiology-Regulatory, Integrative, and Comparative Physiology*, *291*, 927-935.

Rowe, L.J., Maddock, K.R., Lonergan, S.M., & Huff-Lonergan, E. (2004). Oxidative environments decrease tenderization of beef steaks through inactivation of μ-calpain. *Journal of Animal Science*, *82*, 3254-3266.

Ryynänen, M., Lampi, A.M., Salo-Väänänen, P., Ollilainen, V., & Piironen, V. (2004). A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals. *Journal of Food Composition and Analysis*, *17*, 749-765.

Samaniego-Sánchez, C., Troncoso-González, A. M., García-Parrilla, M. C., Quesada-Granados, J. J., López-García de la Serrana, H., & López-Martínez, M. C. (2007). Different radical scavenging tests in virgin olive oil and their relation to the total phenol content. *Analytical Chimica Acta*, *593*, 103-107.

Santé-Lhoutellier, V., Engel, E., & Gatellier, Ph. (2008). Assessment of the influence of diet on lamb meat oxidation. *Food Chemistry*, *109*, 573-579.

Savary-Auzeloux, I., Durand, D., Gruffat, D., Bauchart, D., & Ortigues-Marty, I. (2008). Food restriction and refeeding in lambs influence muscle antioxidant status. *Animal*, *2*, 738-745.

Scollan, N. D., Hocquette, J-F., Nuernberg, K., Dannenberger, D., Richardson, R. I., & Maloney, A. (2006). Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Science, 74,* 17-33.

Scollan, N.D., Choi, N.J., Kurt, E., Fisher, A.V., Enser, M., & Wood, J.D. (2001). Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. *British Journal of Nutrition*, *85*, 115-124.

Serrano, J., Goñi, I., & Saura-Calixto, F. (2007). Food antioxidant capacity determined by chemical methods may underestimate the physiological antioxidant capacity. *Food Research International*, *40*, 15–21.

Shantha, N.C., Crum, A.D., & Decker, E.A. (1994). Evaluation of conjugated linoleic acid concentrations in cooked beef. *Journal of Agricultural and Food Chemistry*, *42* (8), 1757-1760.

Shingfield, K.J., Bernard, L., Leroux, C., & Chilliard, Y. (2010). Role of trans fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. *Animal*, *4*, 1140-1166.

Simopoulus, A.P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medizine, 23*3, 674-688.

Smith, S.B., Gill, Cl.A., Lunt, D.K., & Brooks, M.A. (2009). Regulation of Fat and Fatty Acid Composition in Beef Cattle. *Asian-Australasian Journal of Animal Sciences*, *22* (9), 1225-1233.

Stevez-Garcia, M., & Cava, R. (2004). Plasmalogens in pork: aldehyde composition and changes in aldehyde profile during refrigerated storage of raw and cooked meat. *Food chemistry, 85* (1), 1-6.

Suresh, D.R., Annam, V., Pratibha, K., & Prasad, B.V. (2009). Total antioxidant capacity – a novel early bio-chemical marker of oxidative stress in HIV infected individuals. *Journal of Biomedical Science*, *16* (61), 1-4.

Terevinto, A., Ramos, A., Castroman, G., Cabrera, M.C., & Saadoun, A. (2010). Oxidative status, *in vitro* iron-induced lipid oxidation and superoxide dismutase, catalase and glutathione peroxidase activities in rhea meat. *Meat Science*, *84*, 706-710.

Therkildsen, M., Houbak, M.B., & Byrne, D.V. (2008). Feeding strategy for improving tenderness has opposite effects in two different muscles. *Meat Science, 80*, 1037-1045.

Thorat, V.N., Suryakar, A.N., Naik, P., & Tiwale, B.M. (2009). Total antioxidant capacity and lipid peroxidation in liver transplantation. *Indian Journal of Clinical Biochemistry*, *24* (1), 102-104.

Tres, A., Bou, R., Codony, R., & Guardiola, F. (2008). Influence of different dietary doses of *n*-3 or *n*-6-rich vegetable fats and a-tocopheryl acetate supplementation on raw and cooked rabbit meat composition and oxidative stability. *Journal of Food Chemistry*, *56*, 7243-7253.

Uauy, R. (2009). Dietary fat quality for optimal health and well-being: overview of recommendations. *Annals of Nutrition and Metabolism*, *54* (1), 2-7.

Wang, C. C., Chu, C.Y., Chu, K.O., Choy, K.W., Khaw, K.S., Rogers, M.S., & Pang, C.P. (2004). Trolox-equivalent antioxidant capacity assay *versus* oxygen radical absorbance capacity assay in plasma. *Clinical Chemistry*, *50*, 952-954.

Warren, H.E., Scollan, N.D., Nute, G.R., Hughes, S.I., Wood, J.D., & Richardson, R.I. (2008). Effects of breed and a concentrate or grass silage diet on beef quality in cattle of 3 ages. II: Meat stability and flavour. *Meat Science, 78,* 270-278.

Waters, S.M., Kelly, J.P., O'Boyle, P., Moloney, A.P., & Kenny, D.A. (2009). Effect of level and duration of dietary *n*-3 polyunsaturated fatty acid supplementation on the transcriptional regulation of {Delta} 9-desaturase in muscle of beef cattle. *Journal of Animal Science*, *87*, 244-252.

Wien, L. (2001). Zellschutz durch anti-oxidantien. Accessed April 20th, 2009. http://www.vitaminlauda.com/file/Zellschutz_si.pdf.

Wilson, P.N., & Osbourn, D.F. (1960). Compensatory growth after undernutrition in mammals and birds. *Biological Reviews*. *35* (3), 324-361.

Willett, W.C. (2007). The role of dietary *n*-6 fatty acids in the prevention of cardiovascular disease. *Journal of Cardiovascular Medicine* (Hagerstown), *8* (1), S42-5

Wistuba, T.J., Kegley, E.B., Apple, J.K., & Rule, D.C. (2007). Feeding feedlot steers fish oil alters the fatty acid composition of adipose and muscle tissue. *Meat Science*, *77*, 196-203.

Wood, J.D., Enser, M., Fisher, A.V., Nute, G.R., Sheard, P.R., Richardson, R.I., Hughes, S.I., & Whittington, F.M. (2008). Fat deposition, fatty acid composition and meat quality: A review. *Meat Science, 78,* 343–358.

Wu, C., Duckett, S.K., Neel, J.P.S., Fontenot, J.P., & Clapham, W.M. (2008). Influence of finishing systems on hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) in beef. *Meat Science, 80,* 662–667.

Xu, C.X., Oh, Y.K., Lee, H.G., Kim, T.G., Li, Z.H., Yin, J.L., Jin, Y.C., Jin, H., Kim, Y.J., Kim, K.H., Yeo, J.M., & Choi, Y.J. (2008). Effect of feeding high-temperature, microtime-treated diets with different lipid sources on conjugated linoleic acid formation in finishing Hanwoo steers. *Journal of Animal Science*, *86* (11), 3033-3044.

Yang, A., Lanari, M.C., Brewster, M., & Tume, RK. (2002). Lipid stability and meat colour of beef from pasture and grain-fed cattle with or without vitamin E supplement. *Meat Science, 60,* 41-50.

Yin, M.C., & Cheng, W.S. (1997). Oxymyoglobin and lipid oxidation in Phosphatidylcholine liposomes retarded by α-tocopherol and ß-carotene. *Journal of Food Science, 62* (6), 1095-1097.

Zhao, S., Wang, J., Song, X., Zhang, X., Ge, C., & Gao, S. (2010). Impact of dietary protein on lipid metabolism related gene expression in porcine adipose tissue. *Nutrition & Metabolism*, *7* (6), 1-13.

Zulueta, A., Esteve, M.J., & Frígola, A. (2009). ORAC and TEAC assays: comparison to measure the antioxidant capacity of food products. *Food Chemistry, 114,* 310–316.

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Publications and presentations

1. **Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls.** Paper published in Meat Science 82 (2009): 365-371.
2. **Antioxidant enzyme activities and antioxidant capacity in *longissimus dorsi* muscle from bulls fed diets rich in polyunsaturated fatty acids.** Paper submitted in Food Chemistry. Revision submitted.
3. **Relationship between lipid peroxidation and antioxidant status in the muscle of German Holstein bulls fed *n*-3 and *n*-6 PUFA-enriched diets.** Journal of Agricultural and Food Chemistry DOI:10.1021/jf101218b.
4. **Exogenous PUFA induction on meat quality, lipid peroxidation and antioxidants in beef muscle**. Abstract presented in University of Rostock and in Leibniz Institute for Farm Animal Biology in Dummerstorf, Deutschland at PhD student day 2009.
5. **Exogenous induction of polyunsaturated fatty acids on lipids, lipid peroxidation, and antioxidant status of beef muscle**. Abstract presented in the Annual Meeting of the German Society for Animal Production e.V. (DGfZ) and the Society for Animal Science e.V.(GfT). 16th and 17 September 2009 in Gießen.
6. **Antioxidative Kapazität im Rindfleisch- Welchen Einfluss haben differente Fütterungsregime und Lagerung?** Abstract presented in the Congress of the German Nutrition Society (DGE), 11-12. March 2010, Jena (Germany) by Dr. Dirk Dannenberger as oral presentation.
7. **Effect of diet and storage on the quality and the antioxidant balance of beef muscle from bulls fed different sources of polyunsaturated fatty acids: Preliminary results.** Abstract presented in X National and III International Congress of Animal Research - ENICIP. Medellin, Colombia.
8. **Antioxidative Kapazität im Rindfleisch - Einfluss von Fütterung und Lagerung**. Review submitted to the Journal `Ernährungs-umschau' 05.07.10.

Appendix

Appendix A. Original published paper

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Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls

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ABSTRACT

This study evaluated the effect of diet on fatty acid profile, vitamins, trace elements, lipid peroxidation, and quality of *longissimus* muscle of German Simmental bulls. The effect of storage on fatty acid profile and vitamins was also included. A control group was fed concentrate including soybean, and maize silage/grass silage. Treatment group I (unrestricted) was fed concentrate including rapeseed, and grass silage. Treatment group II (restricted) was fed like treatment group I with a feed restriction period. The treatment diet was not effective to give similar daily live weight gain to the control diet, but it was successful in improving beef fatty acid composition without affecting tenderness and colour (under unrestricted conditions). There were no differences in vitamins and cooking loss, but selenium decreased in treatment groups. Stimulated lipid peroxidation, in samples taken immediately post-mortem, was higher in treatment groups. Polyunsaturated fatty acids decreased, saturated fatty acids and intramuscular fat increased after 14 days of storage while vitamins had no significant reduction.

1. Introduction

A high incidence of mortality in Europe resulting from cardiovascular problems associated with the actual diet has been recently found by Müller-Nordhorn, Binting, Roll, and Willich (2008). Studies like this have increased the interest of consumers in healthy food. For beef, much attention has been given to lipids, nutrition being the major route for increasing the content of beneficial fatty acids in beef (Scollan et al., 2006). It is well known that part of dietary 18:2*n*-6 and 18:3*n*-3 fatty acids can escape ruminal biohydrogenation and go on to be deposited in the tissues. However, most of them are extensively metabolized and biohydrogenated in the rumen (Harfoot & Hazlewood, 1997). The rumen microbial ecosystem yields not only the final product: stearic acid (18:0), but a range of biohydrogenation intermediates such as monounsaturated *trans*, cis and conjugated fatty acids (Jouany, Lassalas, Doreau, & Glasser, 2007). Some of these intermediates are transported via duodenal digesta and deposited finally in the tissues (Scollan et al., 2006). Therefore, it is really important that producers, industry and researchers generate alternatives to improve beef quality under the actual beef production conditions. A common diet used for beef cattle production under indoor conditions in Europe consists of maize silage:grass silage with a higher proportion of maize silage, and a concentrate including soybean. A high inclusion of maize silage in beef cattle diets has been related to a high amount of linoleic acid (18:2*n*-6), high *n*-6/*n*-3 ratio and low vitamin E contents in beef (O'Sullivan et al., 2002). In the same way, the use of grain and soybean has also been related with an increase of *n*-6 PUFA and an unbalance in the *n*-6/*n*-3 ratio (Kim, Adesogan, Badinga, & Staples, 2007) which is not desired for human health. Hence, the first purpose of this study was to evaluate an alternative diet in order to increase beneficial fatty acids in beef, without affecting other parameters of beef quality. Likewise, it was considered that just as the improvement of beef quality using different feeding strategies is important for actual and future beef production systems, the maintenance of quality after slaughter is necessary in order to guarantee quality to the consumers. This study had two objectives: (i) to evaluate fatty acid profile, trace elements, fat soluble vitamins, lipid peroxidation, and quality of *longissimus* muscle of German Simmental bulls comparing control animals fed concentrate including soybean and a mixture of maize silage and grass silage (70:30), versus restricted and unrestricted animals fed concentrate, including rapeseed and only grass silage, (ii) to investigate the effects of storage (14 days of storage versus 24 h post-mortem) on fatty acid profile and fat soluble vitamins.

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Appendix B. Original published paper

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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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Appendix C. Abstract presented in University of Rostock and in Leibniz Institute for Farm Animal Biology in Dummerstorf, Deutschland at PhD student day.

Exogenous PUFA induction on meat quality, lipid peroxidation and antioxidants in beef muscle

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Abstract

This study evaluated the effect of diet on fatty acid profile, vitamins, trace elements, lipid peroxidation, and quality of *longissimus* muscle of German Simmental bulls. The effect of storage on fatty acid profile and vitamins was also included. Control group fed concentrate including soybean, and maize silage/grass silage. Treatment group I (unrestricted) fed concentrate including rapeseed, and grass silage. Treatment group II (restricted) fed like treatment group I with a feeding restriction period. Treatment diet was not effective to get similar daily live weight gain than control diet, but it was successful to improve beef fatty acid composition without affecting tenderness and colour (under unrestricted conditions). There were not differences in vitamins and cooking loss, but selenium decreased in treatment groups. Lipid peroxidation was higher in treatment groups. Polyunsaturated fatty acids decreased, saturated fatty acids and intramuscular fat increased after 14 days of storage while vitamins had no significant reduction.

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Appendix D. Abstract presented in the Annual Meeting of the German Society for Animal Production e.V. (DGfZ) and the Society for Animal Science e.V.(GfT). 16th and 17 September 2009 in Gießen

B24

Exogenous induction of polyunsaturated fatty acids on lipids, lipid peroxidation and antioxidant status of beef muscle

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1. Introduction

Research about PUFA supplementation in beef cattle diets has increased during the last decade trying to improve the nutritional and health value of beef. It is known that PUFA are highly susceptible to lipid peroxidation by reactive species coming from endogenous and exogenous sources. Actually, the balance and the interaction between anti- and prooxidants especially in beef cattle by exogenous PUFA induction is one subject of international research. The objective of this study was to evaluate the effect of a treatment diet with higher amount of *n*-3 PUFA (with and without feeding restriction) compared with a control diet with higher amount of n-6 PUFA, on the fatty acid composition and on the interaction of lipid peroxidation and antioxidant status of fresh muscle from German Simmental bulls. The effect of storage after 14 days on fatty acids, lipid-soluble vitamins, and antioxidant capacity (AOC) was also evaluated.

2. Materials and methods

It was used 25 male German Simmental in an indoor experiment. Control group (n = 9) were daily fed maize silage/grass silage (70/30, *ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including soybean. Treatment group I, consisted of unrestricted animals (n = 7), fed grass silage (*ad libitum*), 1 kg of molasses, 1 kg of hay, and concentrate including rapeseed in the same proportion as the control group. Treatment group II, consisted of restricted animals (n = 9), fed as treatment group I with a restriction of 1 kg of concentrate (50%) per day during the first 112 days of the fattening period. Fatty acid composition of muscle was analyzed as described previously (Nuemberg et al., 2002). Retinol (vitamin A), tocopherol isomers, and ß-carotene were analyzed as recently described (Mahecha et al., 2009). Selected trace element contents (Se, Cu, Fe and Zn) were performed as described by Dannenberger et al. (2007). TBARS was measured at 0, 5, 15, 30, 60 and 120 minutes according to the method described by Kuechenmeister et al. (1999). CAT, GSH-Px, and SOD activities were measured according to the methods described by Aebi (1974), Hernandez et al. (2004), and Marklund & Marklund (1974), respectively. AOC of hydrophilic and lipophilic antioxidants was evaluated in fresh and stored beef muscle using the Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) assay at reaction times of 5, 30, and 60 minutes. Based on this, the methods are called as $FRAP_{water}$, $FRAP_{lipid}$, $TEAC_{water}$, and $TEAC_{lipid}$ assays. FRAP assay was carried out according to Benzie and Strain (1996), and values were corrected by the endogenous iron content of the muscle samples. TEAC assay was carried out according to Hubbermann et al (unpublished results). All data were analyzed by the least-squares means method using Proc MIXED of SAS ®. Significant differences, at $p \leq 0.05$, were determined using the Tukey test.

3. Results and discussion

Treatment diet was successful in improving the fatty acid composition in *longissimus* muscle (Mahecha et al. 2009). CAT and SOD activities were also increased in treatment groups. There were no differences in AOC (Table 1), vitamin contents, and in most of trace element contents, except selenium that decreased in treatment diets (Mahecha et al. 2009). Stimulated lipid peroxidation was higher in treatment groups (Figure 1). PUFA were lower and saturated fatty acids (SFA) were higher (Mahecha et al. 2009), and AOC changed according to the method, after 14 days of storage (Table 1) while vitamins had no significant reduction (Mahecha et al. 2009). AOC increased over the time (Figure 2). The results of this study demonstrated that a diet comprised of grass silage and concentrate including rapeseed had a positive impact on beef fat quality. However, the higher lipid peroxidation with treatment diet could indicate that the protection conferred by this diet was not enough to compensate the higher offered PUFA levels. 30 minutes were found like a minimum reaction time recommended for AOC using both assays. Generally, AOC values of the hydrophilic antioxidants were significantly higher compared to the values of the lipophilic antioxidants.

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Table 1. AOC of fresh and stored samples in each treatment measured by FRAP (Fe^{2+} equivalents in mmol per g muscle) and TEAC (Trolox equivalents in mmol per g muscle) assays

	Control Group		Treatment groups			
			I Unrestricted		II Restricted	
	24 h	14 d	24h	14d	24h	14d
FRAPwater	2.41a	2.05b	2.45a	1.99b	2.64a	2.05b
FRAPLipid	1.03 ac	0.75b	0.98ab	0.82ab	1.06a	0.79cb
TEACwater	10.14a	12.27b	9.68a	11.66b	9.75a	11.95b
TEACLipid	4.35a	5.63b	4.30a	5.41b	4.37a	5.59b

Means with different letters in the same row are significantly different at P<0.05

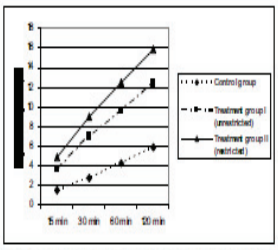

Figure 1. Lipid peroxidation of *longissimus* muscle of German Simmental bulls measured as TBARS at different times. Significant differences between control group and both treatment groups. Reference: Mahecha et al. (2009)

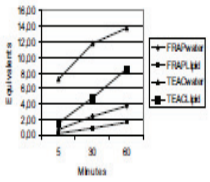

Figure 2. AOC in muscle at different reaction times

4. Summary

A treatment diet with a higher amount of *n*-3 PUFA, composed by grass silage and concentrate including rapeseed (with and without feeding restriction) was compared with a control diet higher in *n*-6 PUFA, composed by a mixture of maize silage/grass silage (70:30) and concentrate including soybean. The effect of diet was evaluated on fatty acid composition, lipid peroxidation, lipid-soluble vitamins, trace elements, endogenous enzymes, and AOC of hydrophilic and lipophilic antioxidants in muscle from German Simmental bulls. Additionally, it was measured the effect of storage on fatty acids, vitamins, and AOC. Fatty acid composition improved, vitamins did not change, and selenium decreased in treatment diet. CAT and SOD activities were significantly higher in treatment groups, and GSH-Px activity was not different. AOC was not affected by the diet. PUFA were lower, saturated fatty acids, and AOC changed according to the method, after 14 days of storage while vitamins had no significant reduction. FRAP and TEAC values were time-depending.

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6. References

Aebi, H.E., 1974: Catalase. Methods for enzymatic analysis. Vol. 1 (pp. 273- 286). Verlag Chemie, Weinheim: HU. Bergmeyer ed.

Benzie, I.F.F., Strain, J.J., 1996: The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP Assay. Anal. Biochem. 239, 70-76.

Dannenberger, D., Reichardt, W., Danier, J., Nürnberg, K., Nürnberg, G., & Ender, K. (2007). Aktuelle Untersuchungen zu Gehalten ausgewählter Spurenelemente im Fleisch deutscher Schweinerassen. Fleischwirtschaft, 87(6), 90–93.

Hernandez, P., Zomenno, L., Arinno, B., & Blasco, A. (2004). Antioxidant, lipolytic and proteolysis enzyme activities in pork meat from different genotypes. Meat Sci. 66, 525-529.

Kuechenmeister, U., Nuernberg, K., Fiedler, I., Kuhn, G., Nuernberg, G., & Ender, K. (1999). Cell injury and meat quality of pig in the time period postmortem from two genotypes susceptible or resistant to malignant hyperthermia. Eur. Food Res. Technol. 206, 97–103.

Mahecha, L., Nuernberg, K., Nuernberg, G., Ender, K., Hagemann, E., & Dannenberger, D. (2009). Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls. Meat Sci. 82, 365–371.

Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469-474.

Nuernberg, K., Nuernberg, G., Ender, K., Lorenz, S., Winkler, K., Rickert, R., et al. (2002). n-3 Fatty acids and conjugated linoleic acids of longissimus muscle in beef cattle. Eur J Lip Sci Tech. 104, 463-471.

Appendix E. Abstract presented in the Congress of the German Nutrition Society (DGE), 11.- 12. March 2010, Jena (Germany)

Antioxidative Kapazität im Rindfleisch - Welchen Einfluss haben differente Fütterungsregime und Lagerung ?

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Im Rahmen eines Fütterungsexperimentes mit Bullen der Rasse Deutsches Fleckvieh (n=25) sollten durch Variation des Futters (Maissilage *vs.* Grassilage) und der Zufuhr exogener Polyenfettsäuren (PUFA) in Form von Soja- und Rapssupplementen Aspekte des Lipid-stoffwechsels und des antioxidativen Status im Rindermuskel (M. *longissimus dorsi*) untersucht werden. Ein Schwerpunkt war die Untersuchung der Wechselwirkungen zwischen Lipidperoxidation und antioxidativer Kapazität (AOC) im Muskel 24 Stunden nach der Schlachtung und nach 14 Tagen Lagerung.

Methoden

Die Methoden zur Bestimmung der antioxidativen Kapazität im Rindermuskel wurden basierend auf den Messprinzipien des TEAC- und des FRAP Assays entwickelt. Beide AOC Assays wurden sowohl im wässrigen als auch im organischen Lösungsmittelextrakt (Aceton, Ethanol und Wasser (5:4:1, v:v:v)) durchgeführt.

Einfluss der Fütterung

Die Ergebnisse des FRAP und des TEAC Assays zeigten, dass sowohl im wässrigen und auch im Lösungsmittelextrakt die unterschiedliche Fütterung keinen Einfluss auf die antioxidative Kapazität im Rindermuskel hatte. Die Aktivitäten im TEAC Assay (wässriger Extrakt) waren höher im Vergleich zum TEAC Assay im Lösungsmittelextrakt. Die Aktivitäten derselben Extrakte lagen im FRAP Assay auf einem niedrigeren Niveau, wenn Trolox zum Vergleich als Referenzsubstanz herangezogen wird.

Einfluss der Lagerung

Die Ergebnisse des TEAC und FRAP Assays zum Einfluss der Lagerung des Rindfleisches zeigten unterschiedliche Effekte. Im TEAC Assay (Lösungsmittelextrakt) der gelagerten Proben wurden signifikant höhere AOC Werte gemessen. Im TEAC Assay (wässriger Extrakt) hatte die Lagerung keinen signifikanten Einfluss auf die AOC, zeigte aber eine Tendenz zu höheren Werten. Im Gegensatz dazu zeigten die Ergebnisse des FRAP Assay, das die Lagerung des Rindfleisches zu einer signifikanten Abnahme der antioxidativen Kapazität, vorrangig beim wässrigen Extrakt, führt.

Appendix F. Abstract presented in X National and III International Congress of Animal Research - ENICIP. Medellin, Colombia

Effect of diet and storage on the quality and the antioxidant balance of beef muscle from bulls fed different sources of polyunsaturated fatty acids: Preliminary results

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In the last decade the international research is focused on improving the nutritional and health value of beef. Much attention has been given on strategies for increasing the content of *n*-3 polyunsaturated fatty acids (PUFA). It's well known that PUFA are highly susceptible to lipid peroxidation. The

objective of this study was to evaluate the effects of diet and storage on the quality and antioxidant balance of the *longissimus* muscle from German Simmental bulls. 25 male German Simmental bulls were included in an indoor experiment. Group I was fed concentrate including soybean, and maize silage/grass silage (70:30) (more *n*-6 PUFA). Group II was fed concentrate including rapeseed and grass silage (more *n*-3 PUFA). Group III was fed like group II with a period of feeding restriction. It has been evaluated the effect of diet on the quality (fatty acid profiles, contents of fat soluble vitamins, trace elements, tenderness, color, and cooking loss), lipid peroxidation, endogenous enzymes activities, and antioxidant capacity (AOC) of muscle. Diet used for groups II and III was successful in improving the fatty acid composition in fresh *longissimus* muscle without affecting tenderness and color (under unrestricted conditions) than diet used for group I. Enzyme activity of catalase and superoxide dismutase also increased in group II and III. There were no differences in vitamin contents, cooking loss, AOC, and in most of trace element contents, except selenium. Stimulated lipid peroxidation was higher in treatment groups. The effect of storage after 14 days on fatty acids, vitamins, and AOC has also been evaluated. PUFA were lower, saturated fatty acids, and intramuscular fat were higher, and AOC changed according to the method, after 14 days of storage while vitamins had no significant reduction.

Curriculum vitae

PERSONAL INFOMATION

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EDUCATION

	School
1976-1981	San Pedro Claver College. Santiago de Cali, Valle, Colombia.
1981-1987	Hispano Americano College. Santiago de Cali, Valle, Colombia.
07.1987	General qualification for university entrance.

	Academic studies
2007-2010	**Doctor of Agriculture (Dr. Agr.)** Faculty of Agricultural and Environmental Sciences. University of Rostock, Germany; Leibzip Institute for Farm Animal Biology of the Farm Animals (FBN), Germany.
1996 - 1998	**Master of Animal Science** National University of Colombia. Palmira, Colombia.
1988 - 1993	**Animal Production Science « Zootechnician»** National University of Colombia. Palmira, Colombia.

Other studies
2007 English as a second language. Malaspina University Canada
2006. Statistical design of experiments. University of Antioquia
2005. Moodle. Tools for online class preparation. University of Antioquia.
2002. Production and cost in dairy cattle.
2000. New topics in ruminant nutrition**.**
2000. Seminary for beginner's professors. University of Antioquia.

OCCUPATIONAL ACTIVITIES

2000 - Currently	Permanent position. University of Antioquia. Professor and Researcher Lines of research: 1. Silvopastoral Systems 2. Sustainable Animal Production Systems 3. Improve of beef production and quality
	Director of Animal Science Master program (2001-2006)
	Professor of: 1. Tropical Animal Production (Master of Animal Science program) 2. Silvopastoral systems for livestock 3. Seminary of Research (Master of Animal Science program) 4. Beef Cattle Production: Undergraduate 5. Races and phenotypic evaluation of beef cattle: Undergraduate 6. Introduction to traditional Species of Animals: Beef Cattle (Undergraduate)
1999-1999	National University of Colombia. Director of the Research farm. (Part-time)
1997-1998	National University of Colombia. Professor of Animal physiology and Animal Eco-physiology (Part-time)
1996 - 1999	Centre for research in sustainable systems of animal production – CIPAV. Researcher of Agroforestry Systems and Livestock (Part-time)
1994 - 1996	Solla S.A. Technical consultant and marketing of food animal products

PRESENTATIONS IN CONGRESS AND PUBLICATIONS

DURING THE LAST 5 YEARS

Poster/oral presentations
DIRK DANNENBERGER, LILIANA MAHECHA, SANDRA KNÖLLER, EVA MARÍA HUBBERMANN, KARIN NUERNBERG, GERD NUERNBERG, K. SCHWARZ. Antioxidative Kapazität im Rindfleisch- Welchen Einfluss haben differente Fütterungsregime und Lagerung ? Jena, Germany. March , 2010.
LILIANA MAHECHA, KARIN NUERNBERG, GERD NUERNBERG, KLAUS ENDER, JÖRG MARTIN, EVA MARIA HUBBERMAN, SANDRA KNOELLER, ERIK CLAEYS, STEFAAN DE SMET, ELKE HAGEMANN, DIRK DANNENBERGER. Effect of diet and storage on the quality and the antioxidant balance of beef muscle from bulls fed different sources of polyunsaturated fatty acids: Preliminary results. ENICIP. Colombia. October, 2009.
LILIANA MAHECHA, KARIN NUERNBERG, GERD NUERNBERG, DIRK DANNENBERGER. Exogenous PUFA induction on meat quality, lipid peroxidation and antioxidants in beef muscle. Leibniz Institute for Farm Animal Biology in Dummerstorf, Germany at PhD student day. April 23rd, 2009.
LILIANA MAHECHA, KARIN NUERNBERG, GERD NUERNBERG, DIRK DANNENBERGER. Exogenous PUFA induction on meat quality, lipid peroxidation and antioxidants in beef muscle. University of Rostock. PhD day. April 24th, 2009.
JOAQUIN ANGULO ARIZALA, SERGIO YEPES, ANGELA MARIA YEPES, GILBERTO BUSTAMANTE, LILIANA MAHECHA LEDESMA, "Evaluation of fruit- vegetable wastes (fv) from a market place as alternative feedstuffs in dairy cattle diets": Spain, Palma de Mayorca. September, 2008. 3rd IMEBE (International Meeting on Environmental Biotechnology & Engineering. Oral Presentation.
JOAQUIN ANGULO ARIZALA, SERGIO YEPES, GILBERTO BUSTAMANTE, ANGELA MARIA YEPES, LILIANA MAHECHA LEDESMA, "Fruit and vegetable waste (fv) from the market places: a potential source for animal feeding?" Spain, Palma de Mayorca. September, 2008. 3rd IMEBE (International Meeting on Environmental Biotechnology & Engineering. Oral Presentation.
CAROLINA MESA, JORGE GALLO, JOAQUIN ANGULO ARIZALA, ZULMA TATIANA RUIZ CORTES, MARTHA OLIVERA ANGEL, LILIANA MAHECHA LEDESMA, "Bypass fat supplementation in zebu cows (*Bos indicus*) in the early postpartum: an alternative to decrease the open days period ?" Hungary. July, 2008. 16 th INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION 2008 *Reproduction in Domestic Animals*, 43 (3). Poster.
ANGULO JOAQUIN, GIRALDO C, MARIO FERNANDO CERON MUNOZ, MARTHA

OLIVERA ANGEL, LILIANA MAHECHA LEDESMA, "Effect of supplementation with bypass fat on maternal ability in beef cows" New Zeeland. 2006. 7th International Congress of ruminant reproduction.

Published papers in Journals

MAHECHA L, DANNENBERGER D, NUERNBERG K, NUERNBERG G, HAGEMANN E, MARTIN J.2010. Relationship between Lipid Peroxidation and Antioxidant Status in the Muscle of German Holstein Bulls Fed n-3 and n-6 PUFA-Enriched Diets. Journal of Agricultural and Food Chemistry. DOI:10.1021/jf101218b

MAHECHA L, NUERNBERG K, NUERNBERG G, ENDER K, HAGEMANN E, DANNENBERGER D. 2009. Effects of diet and storage on fatty acid profile, micronutrients and quality of muscle from German Simmental bulls. Meat Science 82 (3): 365-371.

ANGULO J, MAHECHA L, OLIVERA M. 2009. Synthesis, composition and modification of the Bovine milk fat: a valuable nutrient for the human health. Rev.MVZ Córdoba 14(3):1856-1866. Review.

L. MAHECHA, J. ANGULO, B. SALAZAR, M. CERÓN , J. GALLO, C. H. MOLINA, E. J MOLINA, J. F. SUÁREZ, J. J. LOPERA, M. OLIVERA. 2008. Supplementation with bypass fat in silvopastoral systems diminishes the ratio of milk saturated/unsaturated fatty acids. Trop Anim Health Prod. 40(3): 209-216.

ANGULO, J, MAHECHA L, CERON M, GIRALDO C, GALLO J, OLIVERA-ANGEL M. 2007. Weight and body condition of Zebu and Angus x Zebu cows in first calving and calves weight, supplemented with rumen protected fat. Revista Orinoquia 11(2): 49-58.

MAHECHA L, ESCOBAR J P, SUÁREZ J F Y RESTREPO L F. 2007: Tithonia diversifolia (hemsl.) Gray (Botón de oro) as forage supplement of Holstein x Zebu cows Livestock Research for Rural Development. 19 (16).
http://www.cipav.org.co/lrrd/lrrd19/2/mahe19016.htm

MAHECHA L, MONSALVE M A Y ARROYAVE J F. 2007: Evaluation of grass availability and growth of Zebu steers in a silvo-pastoral system based on Eucalyptus tereticornis and Panicum maximum in the San Sebastian reforestation program in Magdalena Department of Colombia. Livestock Research for Rural Development. 19 (94). http://www.cipav.org.co/lrrd/lrrd19/7/mahe19094.htm

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.