Je kunt meer dan je durft

(BZN)

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ELS VOSSEN

STRATEGIES TO REDUCE NITRITE AND TO INCREASE N-3

POLYUNSATURATED FATTY ACIDS IN MEAT AND MEAT PRODUCTS:

OXIDATIVE STABILITY AND NUTRITIONAL QUALITY

Thesis submitted in fulfilment of the requirements

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Dutch translation of the title:

STRATEGIEËN VOOR HET VERLAGEN VAN NITRIET EN HET VERHOGEN VAN N-3 POLY-ONVERZADIGDE IN VLEESPRODUCTEN: OXIDATIEVE STABILITEIT EN NUTRITIONELE KWALITEIT

AA   ascorbic acid, arachidonic acid

AAS   α-aminoadipic semialdehyde

ALA   α-linolenic acid

CAT   catalase

DHA   docosahexaenoic acid

DHAA   dehydroascorbic acid

DPA   docosapentaenoic acid

EPA   eicosapentaenoic acid

FRAP   ferric reducing ability of plasma

GGS   γ-glutamic semialdehyde

GSH-Px   glutathione peroxidase activity

HDL   high density lipoprotein

LA   linoleic acid

LDL   low density lipoprotein

MDA   malondialdehyde

MUFA   monounsaturated fatty acids

PE   pre-converted extract

PI   peroxidisability index

PUFA   polyunsaturated fatty acids

RC   *Rosa canina* L., dog rose

SA   sodium ascorbate

SFA   saturated fatty acids

SN   sodium nitrite

TAG   triacylglycerols

TBARS   thiobarbituric acid reactive substances

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GENERAL BACKGROUND

Industrialised countries are confronted with a large incidence of coronary heart disease, stroke, hypertension, diabetes and cancers, which are the most common causes of death. While the main factors age, sex and genetic susceptibility are unchangeable, many of the risks associated with these factors can be changed. It is now well established that inadequate dietary habits and physical inactivity are the major preventable risks for the occurrence of chronic diseases. Nutrition is coming to the front as a major modifiable determinant of chronic disease, with scientific evidence increasingly supporting the view that alterations in diet have strong effects, both positive and negative, on health throughout life (World Health Organization, 2003).

Despite attempts to provide education about healthier eating patterns, there are several barriers such as a lack of interest towards changing one's diet, or concerns about having to compromise on taste or enjoyment (Kearney & McElhone, 1999). In this view, enhancing the composition of popular food products is applied worldwide and its contribution to a healthier society is accepted. Hereby, increasing the content of health-promoting n-3 polyunsaturated fatty acids (PUFA) and lowering the nitrite content of meat products are two aspects that are discussed in this dissertation, with focus on the sensory quality of these enhanced meat products.

The concept meat quality is multi-factorial and covers many attributes. The criteria consumers associate with the quality of meat are: nutritional value, wholesomeness, freshness, leanness, juiciness, taste and tenderness (Grunert, 1997). In this PhD research the attributes colour, oxidative stability, texture and taste are considered. In general, the oxidation-reduction process is defined as a chemical reaction in which one or more electrons are transferred from one atom or molecule to another. Lipid oxidation and pigment oxidation are recognised as the most important causes of quality deterioration of both fresh and processed meat during storage and are extensively studied (see reviews e.g. Gray et al., 1996; Pegg & Shahidi, 1997; Morrissey et al.,

1998; Mancini & Hunt, 2005). Only recently, the importance of protein oxidation on the quality of muscle food has been acknowledged (reviewed by Lund et al., 2011; Zhang et al., 2012).

1. Nitrite

Nitrite and health

Ingested nitrite can form nitric oxide within the human body. Nitric oxide is identified as one of the most important cellular signalling mechanisms. It signals arteries to relax and expand, immune cells to kill bacteria, and brain cells to communicate with each other (Parthasarathy & Bryan, 2012). The lack of nitric oxide production can lead to hypertension, atherosclerosis, heart failure, and thrombosis leading to heart attack and stroke. Remarkably, all of these conditions have been shown to be positively affected by dietary nitrite interventions (Lundberg et al., 2008). On the other hand, potentially carcinogenic N-nitroso compounds can be formed from nitrite in the presence of low molecular weight secondary amines. The International Agency for Research on Cancer (2008) concluded that "ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans" (Ferlay et al., 2008). The World Cancer Research Fund (2011) recommended to avoid processed meat based on a meta-analysis of cohort studies showing increased risk of colorectal cancer with increased intake of processed meats (Demeyer et al., 2008). Although it is not clear to what extent the nitrite used in curing salt is related to this issue, public concern raises and a shift in consumers preferences towards the consumption of natural foods is observed (Sebranek & Bacus, 2007). As a result, the meat producers and meat scientists are challenged to search for reliable alternatives for nitrite.

Nitrite and meat

Preservation of meat with nitrite has become important in controlling meat against *Clostridium botulinum* and in producing safe and palatable meat products with good keeping properties even at ambient temperature (Skibsted, 2011). When added to a meat system, nitrite has different fates. It is partially oxidized to nitrate by sequestering oxygen or reduced to nitric oxide that subsequently binds to different substances, such as myoglobin and other proteins (Honikel, 2008). One of the most noteworthy properties of nitrite is its ability to produce the characteristic pink colour of cooked cured meat products. Actually, it is the nitric oxide that reacts with the raw meat pigment to produce red nitric oxide myoglobin, which converts to pink nitrosylmyochromogen upon cooking (Pegg & Shahidi, 1997). The antioxidant effect of nitrite is likely due to the same mechanisms responsible for cured colour development, involving reactions with heme proteins and metal ions, chelation of free radicals by nitric oxide, and the formation of nitroso- and nitrosyl-compounds having antioxidant properties (Honikel, 2008). Cured meat flavour continues to be one of the least understood aspects of nitrite curing. Nitrite chemistry and associated reactions likely play a role in the formation of the unique flavour, however, the specific compounds involved, are still not yet known (Sindelar & Milkowski, 2011). The antimicrobial action of nitrite against *Clostridium botulinum* involves activity against the iron-containing enzymes ferredoxin and pyruvate oxidoreductase. Various other bacteria are also inhibited by nitrite, although specific modes of action are unclear (Simpson & Sofos, 2009).

The ingoing and residual nitrite content of cured meat products is restricted by government regulations, because of the possible formation of harmful N-nitroso compounds. An all-round alternative for nitrite, including all four features of nitrite has not yet been found.

2. n-3 Polyunsaturated fatty acids

n-3 PUFA and health

Fatty acids can be considered the defining components of lipids. Their structure, biochemistry and functions have been extensively studied and reviewed (AOCS Lipid Library, 2014). Two principal families of PUFA, n-6 and n-3, are derived biosynthetically from respectively linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3). LA is the precursor of the bioactive arachidonic acid (AA, C20:4n-6), while ALA is the precursor of the bioactive eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Both precursors are converted to their long chain metabolites by a series of desaturation and elongation steps and share common enzymes for these metabolic transformations (Williams, 2000). LA and ALA are strictly essential fatty acids, as they can not be synthesized *de novo* and must be obtained from the diet. In addition, as the conversion of ALA towards EPA and especially DHA is low in humans (Hussein et al., 2005), it is advised to consume foods rich in EPA and DHA, such as fatty fish (Aranceta & Pérez-Rodrigo, 2012). However, the intake of fish is low in Western countries and the consumption of n-3 PUFA from terrestrial animal products (e.g. meat, eggs) may be important (Howe et al., 2006).

Besides their contribution to energy supply and their structural function as part of the phospholipid bilayer in cell membranes, EPA and DHA are used to produce hormone-like substances, eicosanoids, which regulate a wide range of biological functions. These functions extend from developmental roles, especially in the nervous system, during infancy to the attainment and maintenance of optimal mental and physical health status throughout adult life. Moreover, these fatty acids have a protective influence on several chronic diseases due to their beneficial cardiovascular, anti-thrombotic, anti-inflammatory and immune-suppressive properties (reviewed by Ruxton et al., 2004; Narayan et al., 2006; Simopoulos, 1999). It is

believed that ALA has limited biological functions and its principal role is being a substrate for synthesis of EPA and DHA (Burdge, 2004). As the dietary intake of EPA and DHA is low and the daily recommendations are often not fulfilled, Mantzioris et al. (2000) proposed that n-3 PUFA enriched foods would provide a solution in achieving the desired biochemical effects of n-3 PUFA without the intake of supplements, or change in dietary habits.

However, one must be cautious when consuming high amounts of PUFA, given their susceptibility to oxidation. Lipid hydroperoxides and their decomposition products may cause damage to proteins, membranes and biological components, thus affecting vital cell functions (Frankel, 1984). It should therefore not be ignored that the beneficial effects of n-3 PUFA may be affected by these oxidative changes.

n-3 PUFA and meat

Although meat only partly contributes to the total fat intake of the diet, optimizing its fatty acid profile deserves attention due to the high meat intake in industrialized countries (Howe et al., 2006). Meat fatty acid content and composition is affected by several genetic (e.g. species, breed and fatness) and environmental factors, amongst which the dietary supply of fatty acids is generally considered to be the most important (Raes et al., 2004). Fat deposition in the pig's carcass is determined by *de novo* fatty acid synthesis and the uptake of exogeneous fatty acids (De Smet et al., 2004). The fatty acids synthesized by the pig are mostly saturated and monounsaturated fatty acids, while the deposition of PUFA occurs if they are included in the diet. The incorporation of n-3 PUFA rich products like grass, rapeseed, algae, linseed and fish oil in livestock feeds, resulting in accumulation of these fatty acids in animal products, received a lot of interest (Wood et al., 2008; Raes et al., 2004). As conversion of ALA to EPA and DHA in animals is low, fish oil and algae in the feed can be used to directly increase the EPA and DHA

content of the meat products. Next to introducing n-3 PUFA in animal feed, these fatty acids can also be added as an ingredient during processing (Valencia et al., 2006).

Including n-3 PUFA in meat might also have adverse effects, as these fatty acids are prone to oxidation. Oxidation processes in meat result in reduced nutritional value and the generation of oxidation products (e.g. malondialdehyde and volatile compounds), leading to off-taste and off-flavour (reviewed by Morrissey et al., 1998). A special challenge when increasing the tissue concentration of n-3 PUFA, is thus to counteract this increased oxidative susceptibility of the meat products. For instance, including antioxidants in animal feed or during processing increases the oxidative stability of meat products (reviewed by e.g.; Decker, 1998; Pokorny et al., 2001).

RESEARCH OBJECTIVES AND THESIS OUTLINE

The aim of this PhD research was to produce healthier meat products that meet consumers' expectations. The overall objective was to change the composition of fresh and processed meat without compromising the sensory quality.

This was done by

[1] lowering the ingoing nitrite level by replacing it with other compounds

[2] increasing the n-3 PUFA content through animal feeding
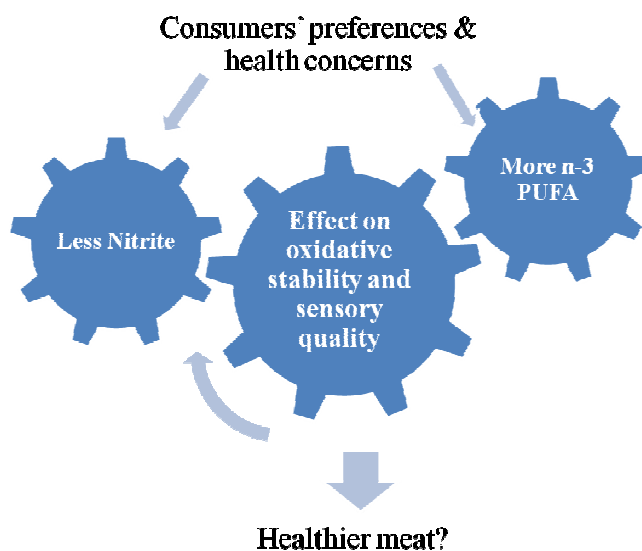
To meet these objectives, several experiments were conducted. **Part I** describes the work performed on lowering the ingoing nitrite in various cooked cured meat products. In **Chapter 1**, frankfurters were produced containing dog rose extracts as a natural antioxidant and no sodium nitrite or sodium ascorbate. Quality parameters such as colour, tenderness, lipid and protein oxidation were assessed. **Chapter 2** deals with the effectiveness of partly replacing sodium nitrite by sodium ascorbate for antioxidant activity in liver pâté, while in **Chapter 3** the use of a pre-converted extract as alternative source for nitrite in liver pâté was investigated. In both chapters the effect of the lower nitrite content on colour, lipid and protein oxidative processes

was assessed. The role of sodium nitrite on protein oxidation has been investigated from a more mechanistic point of view in **Chapter 4** using two meat model systems**.** Furthermore, a marker for protein nitration, 3-nitrotyrosine, was introduced in meat.

In **Part II,** research concerning the increase of n-3 PUFA in meat products and its effect on oxidative stability and nutritional quality is given. For this study, different n-3 PUFA sources (linseed oil, fish oil and dried microalgae) were added to pig feed and the effect on the fatty acid profile (**Chapter 5**) and sensory quality (**Chapter 6**) of fresh loin, dry fermented sausage and long ripened dry cured ham was investigated. In **Chapter 7,** the effect of supplementing supra-nutritional levels of α-tocopherol in n-3 PUFA enriched pig feed was described. It was investigated if these high α-tocopherol doses affected the α-tocopherol content in fresh loin and dry fermented sausages and whether the oxidative stability of these meat products was improved. To explore the effect of n-3 PUFA enriched meat on the health status, cooked n-3 PUFA enriched loin was administered to rabbits and the blood lipids, oxidative status and atherosclerosis were assessed (**Chapter 8**). To conclude, a general discussion and future prospects are given in **Chapter 9.**

Part I	**Lowering the ingoing nitrite dose**	**Part II**	**Increasing the n-3 PUFA content**
CH1	With a dog rose extract	**CH5**	Role of n-3 PUFA source
CH2	With sodium ascorbate	**CH6**	Effect on sensory quality
CH3	With a pre-converted extract	**CH7**	Role of α-tocopherol in feed
CH4	Nitrite and protein oxidation?	**CH8**	Effect on health
	CH9	General discussion and future prospects	

The specific null hypotheses that were formulated for the own research and their relation to the chapters are presented the Table 1.

Table 1. Specific null hypotheses of the own research and their relation to the chapters

	Hypothesis	**Chapter**
H1	Lowering the ingoing nitrite doses in meat products compromises colour formation	1,2,3
H2	Lowering the ingoing nitrite doses in meat products compromises colour stability	1,2,3
H3	Lowering the ingoing nitrite doses in meat products decreases lipid oxidative stability	1,2,3
H4	Lowering the ingoing nitrite doses in meat products decreases protein oxidative stability	1,2,3
H5	The antioxidant role of nitrite in meat products can be compensated by the use of other food additives	1,2,3
H6	Sodium nitrite can be used as an antioxidant against protein oxidation	4
H7	3-nitrotyrosine is a good marker for protein nitration in meat products	4
H8	The efficacy to increase the n-3 PUFA concentration of meat products depends on the n-3 PUFA source in the feed	5
H9	Supplementation of microalgae in pig feed is a suitable and sustainable alternative to fish oil for the production of pork products enriched in EPA and DHA	5,6
H10	Increasing the n-3 PUFA content of meat products compromises its oxidative stability	6
H11	Dietary α-tocopherol supplementation at high levels reduces oxidation processes in n-3 PUFA enriched dry fermented sausages	7
H12	Increasing EPA and DHA concentrations in plasma and other tissues depends on the type of n-3 PUFA in the n-3 PUFA enriched meat	8
H13	Consumption of n-3 PUFA enriched meat improves the health status	8

LOWERING THE INGOING NITRITE LEVEL

People are advised to reduce their intake of nitrite for health reasons.

Can a dog rose extract replace sodium nitrite in frankfurters?

DOG ROSE (*ROSA CANINA* L.) AS A FUNCTIONAL INGREDIENT IN PORCINE FRANKFURTERS WITHOUT ADDED SODIUM ASCORBATE AND SODIUM NITRITE

ABSTRACT

The effect of dog rose (*Rosa canina* L.; RC), rich in polyphenols and ascorbic acid, on lipid and protein oxidation, colour stability and texture of frankfurters is investigated. Four treatments were prepared: with 5 or 30 g/kg RC extract and without sodium ascorbate and sodium nitrite (5RC and 30RC, respectively), a positive control (with sodium ascorbate and sodium nitrite; PC) and a negative control (without sodium ascorbate, sodium nitrite or RC extract; NC). Hexanal values were much higher throughout storage in NC compared to RC and PC frankfurters ($P<0.001$). The RC extracts protected against protein oxidation, but not as efficiently as PC ($P<0.05$). In the RC treated frankfurters, lower a* values were measured compared to PC due to the lack of sodium nitrite. In conclusion, dog rose can act as a natural antioxidant in frankfurters, but not as full replacer for sodium nitrite.

INTRODUCTION

Lipid and myoglobin oxidation is a well-known phenomenon in meat products, which results in discolouration, off-odours and off-flavours during storage (Morrissey et al., 1998). Recently, also protein oxidation has been linked with impared meat quality, such as loss in juiciness and increased toughness of meat (Lund et al., 2011). Oxidation leading to degradation of lipids, proteins and pigments is one of the primary mechanisms of meat deterioration and can be prevented by including antioxidants in meat products. However, the increased public concern over the safety and toxicity of synthetic additives, challenges the meat industry to find natural alternatives.

Natural alternatives can be antioxidant-containing extracts from herbs and spices (Yanishlieva et al., 2006). The main components contributing to the antioxidant effect of these extracts are phenolic compounds, due to their hydrogen-donating capacity and metal-chelating potential (Rice-evans et al., 1995). The rose hips of dog rose (*Rosa canina* L., RC) are rich in phenolic compounds and ascorbic acid (Demir & Ízcan, 2001) and are therefore believed to be a potential natural antioxidant. According to Ganhão et al. (2010a), the main phenolic compounds present in the RC extracts are procyanidins and catechins. In fact, RC extracts have shown high antioxidant activities *in vitro*, with water extracts having greater antioxidant activities against the DPPH and ABTS radicals compared to methanolic or ethanolic extracts (Ganhão et al., 2010a). The use of 30 g *Rosa canina* in an extract that was added to porcine burger patties has resulted in positive effects on improving colour stability, texture properties and on delaying lipid and protein oxidation (Ganhão et al., 2010b). However, the relative efficiency of phenolic-rich extracts when applied in different food matrices can not be predicted even for very well-characterised extracts (Nissen et al., 2004). Therefore, although RC has shown some promising beneficial effects on burger patties, the use of RC in different meat products and different doses needs to be further investigated.

Among other synthetic additives, also the use of sodium nitrite should be revised according to the consumers' opinion. However, this ingredient is very important for the meat industry as it plays a key role in colour development, fat oxidation, flavour and microbiological safety. It is particularly important for cooked meat products, such as frankfurters, as their characteristic pink colour originates from nitrosylhemochromogen, a reaction product of nitrite and denatured myoglobin (Pegg & Shahidi, 1997). However, the potential health risks related to the residual nitrite levels and the formation of harmful N-nitrosamines in meat and meat products demand for a significant decrease in the use of sodium nitrite (Honikel, 2008). As RC contains considerable amounts of nitrates (Cakilcioglu & Khatun, 2011), replacing sodium nitrite by nitrate could result in lower residual nitrite concentrations, reducing also the risk of N-nitrosamine formation during ingestion. In addition, as RC also contains high amounts of ascorbic acid, its use could result in less sodium ascorbate to be added during manufacturing.

The objective of this research is to investigate the potential of RC as functional ingredient in porcine frankfurters without added sodium ascorbate and sodium nitrite in terms of texture and colour, lipid and protein oxidative stability.

MATERIALS AND METHODS

1. Extraction of dog rose

Fruits of dog rose (*Rosa canina* L., RC) were collected at full ripeness in June in the Cáceres region (Spain) and immediately frozen at -80 ºC. For the extraction, whole fruits were ground and 5 or 30 g were weighted for low and high concentrated frankfurters, respectively. The ground fruits were homogenized 1:4 (w/v) in distilled water using an Ultra-Turrax. Subsequently, the homogenates were centrifuged (1400 g, 7 min, 4 °C) and the supernatant was filtered and collected. The residue was re-extracted once more with distilled water (1:2 w/v) following the

procedure previously described and the filtered supernatant was combined with the first supernatant. Finally, distilled water was added to the residue, shaken by hand, filtered and an amount of that filtrate was added to the combined supernatant until 180 ml of extract was obtained. The total phenolic content and antioxidant capacity using the 1,1– diphenyl-2-picryl hydrazyl radical assay (see below) were determined and the extracts were stored in refrigeration until the manufacturing of the frankfurters (less than 24 hours).

2. Manufacture of frankfurters

The experimental frankfurters were manufactured in a pilot plant and the same formulation was used for all frankfurters. For each treatment 1 kg of frankfurters was prepared. The basic recipe was as follows (g/kg raw batter): 700 g porcine meat, 100 g backfat, 180 g distilled water or extract, 20 g sodium chloride and 5 g sodium di- and tri-phosphates (all from ANVISA, Madrid, Spain). Four different types of frankfurters were considered: a negative control (NC) consisting of a basic recipe, without sodium nitrite nor sodium ascorbate; a positive control (PC) consisting of a basic recipe with 0. 1 g/kg sodium nitrite and 0.5 g/kg sodium ascorbate and 2 experimental frankfurters (5RC and 30RC) manufactured with a basic recipe, without sodium nitrite nor sodium ascorbate and, and to which 180 g of *Rosa canina* L. extract (from 5 g or 30 g whole fruits, respectively, see extraction procedure) was added instead of 180 g distilled water. The meat was chopped into small cubes (1 cm^3) and mixed with the sodium chloride (and for PC also sodium nitrite and sodium ascorbate). Then, the meat was minced in a cutter (Stephan UMC 5 Electronic) for 2 min at 2000 g, together with water or extract. After that, the fat was added and minced for 4 min until a homogeneous raw batter was obtained. Finally, the mixture was stuffed into 18 mm diameter cellulose casings, hand-linked at 15 cm intervals and given a thermal treatment for 30 min in a hot water bath (70 °C). After cooling in an ice bath, the frankfurters were wrapped with an oxygen permeable polyvinylchloride film, dispensed in polypropylene trays and subsequently stored for 60 days at 2 ºC in the dark. At each sampling day (days 1, 20,

40 and 60), four frankfurters per treatment were taken out of the refrigerator. A portion of the frankfurters was used for colour and texture measurements and the remainders were frozen at -80 ℃ until analysis.

3. Total phenolics content and antioxidant activity of the extracts

The Folin Ciocalteau reagent was used for the quantification of total phenolics present in the RC extracts, as described by Turkmen et al. (2006) with minor modifications as follows: 0.2 ml extract was mixed with 1 ml of Folin Ciocalteau reagent (10% in distilled water). After 5 min, 0.8 ml of sodium carbonate (7.5% in distilled water) was added and the samples were allowed to stand for 2 h at room temperature in the darkness. The absorbance was measured at 740 nm using a spectrophotometer. A standard curve with gallic acid was used for quantification. Results were expressed as mg of gallic acid equivalents (GAE) per ml extract and analysed in duplicate.

The antioxidant activity of the RC extracts was evaluated by using the 1,1– diphenyl-2-picryl hydrazyl radical (DPPH) assay (Kähkönen & Heinonen, 2003). Briefly, aliquots of 0.033 ml were mixed with 2.0 ml DPPH solution (6×10^{-5} M in methanol). The reaction mixture was stirred and kept in the dark for 6 min at room temperature. The absorbance was measured spectrophotometrically at 517 nm using methanol as a blank. The antioxidant activity against the DPPH radical is expressed as percentage of radicals scavenged after 6 min reaction time (%) and analysed in duplicate.

4. Proximate composition of the frankfurters

Moisture and crude protein content of the frankfurters were determined (AOAC, 2000) and the method of Folch et al. (1957) was used for isolating the fat. Analyses were carried out in duplicate and results are expressed as g/100g frankfurter.

5. Volatile compounds

Lipid oxidation was assessed by determining the lipid-derived volatiles hexanal, heptanal, octanal and nonanal according to Estévez et al. (2003). One gram of homogenized frankfurter was placed in a 2.5 ml vial and the SPME fibre (divinyl-benzene–carboxen–polydimethylxilosane, 50/30 µm) was exposed to the headspace while the sample equilibrated during 30 min immersed in water at 37 °C. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl–95% dimethyl polysiloxane column (30 m, 0.25 mm id., 1.0 µm film thickness; Restek). Compounds were positively identified by comparing their mass spectra with those from standard compounds run on the same conditions. The area of each peak was integrated using ChemStation software and the total peak area was used as an indicator of lipid-derived volatile generated from the samples. Samples were analysed in quadruplicate and results are provided in arbitrary area units (AAU $\times 10^6$).

6. Analysis of α-aminoadipic and γ-glutamic semialdehydes

The protein oxidation products α-aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) were analysed according to Utrera, Morcuende, Rodriguez-Carpena, & Estévez (2011). Briefly, the frankfurters were minced and subsequently homogenized 1:10 (w/v) in 10 mM phosphate buffer containing 0.6 M NaCl. Aliquots of 0.2 ml were taken and proteins were precipitated twice using trichloroacetic acid (10%). Then, protein carbonyl groups were derivatized using: 250 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) containing 1% sodium dodecyl sulfate and 1 mM diethylenetriaminepentaacetic acid, 250 mM MES buffer containing 50 mM p-amino benzoic acid (ABA) and 250 mM MES buffer containing 100 mM NaCNBH$_3$. The mixture was incubated at 37°C for 90 min and the proteins were subsequently precipitated and simultaneously washed. Afterwards, the precipitates were hydrolysed with 6 N HCl at 110 °C for 18 h and the hydrolysates were dried *in vacuo* at 40 °C

using a Savant speed-vac concentrator. Hydrolysates were finally reconstituted with 0.2 ml Milli-Q water and analysed using HPLC (15 cm × 4.6 mm × 5 µm COSMOSIL 5C18-AR-II RP-HPLC column) with fluorescence detection (excitation and emission wavelength of 283 and 350 nm respectively). The mobile phase was a mixture of 50 mM sodium acetate buffer (pH 5.4) and acetonitrile, varying gradually the acetonitrile concentration from 0% to 8% at a flow rate of 1.0 ml/min. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from standard AAS and GGS (synthesised *in vitro* according to Akagawa et al. (2006)). Samples were analysed in quadruplicate and results are expressed as nmol carbonyls/mg protein as quantified using an ABA standard curve.

7. Colour measurements

Colour measurements were performed in fivefold on the surface of the frankfurters using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ), which consists of a measuring head (CR-300), with an 8 mm diameter measuring area and a data processor (DP-301). Colour measurements were made at room temperature with illuminant D_{65} and a 0° angle observer at days 1, 20, 40 and 60 of chilled storage. The L*, a* and b* values (CIE L*a*b* colour system) were assessed as a measure of respectively lightness, redness and yellowness.

8. Texture measurements

Texture profile analysis was carried out at room temperature with a Texture Analyser TA-XT2i (Stable Micro Systems, Surrey, UK). Nine samples (height 2.0 cm) of each treatment were taken and subjected to a two-cycle compression test. The samples were compressed to 40% of their original height with a cylindrical probe of 5 cm diameter and a cross-head speed of 5 mm/s. Following parameters were determined according to descriptions by Bourne (1982): Hardness (g)

is the maximum force required to compress the sample; adhesiveness (g×s) is the work necessary to pull the compressing plunger away from the sample; chewiness (g) is the work needed to masticate the sample for swallowing; springiness (dimensionless) is the ability of the sample to recover its original shape after the deforming force is removed; cohesiveness (dimensionless) is the extent to which the sample could be deformed prior to rupture; resilience (dimensionless) is how well the product regains its original height, measured on the first withdrawal of the cylinder.

9. Statistical analysis

The data were analysed using the general linear model ANOVA procedure considering 'storage day' and 'treatment' as independent variables. Mean differences between groups were tested using Tukey's post hoc test operating at a 5% level of significance. All the statistical analyses were carried out by SPSS for Windows (15.0).

RESULTS

The total phenolic content of the extracts was 109 ± 3 and 554 ± 2 mg GAE/100 ml extract for 5RC and 30RC respectively. The antioxidant activity against the DPPH radical was 42.8 ± 6.0 and 76.0 ± 4.3 % for the 5RC and 30RC extracts respectively.

The moisture, fat and protein content of the frankfurters was 70.0 ± 0.7 g/100g, 9.9 ± 0.6 g/100g and 18.6 ± 0.9 g/100g frankfurter, respectively. No significant differences between treatments were found.

The results regarding lipid oxidation in frankfurters during chilled storage are summarised in Table 1.1. A clear antioxidant effect of RC was observed since up to tenfold lower hexanal values were found in frankfurters treated with RC compared to NC counterparts ($P<0.001$). Also the heptanal and octanal values were lower in the RC frankfurters compared to NC across storage days ($P<0.001$), whereas the nonanal content was not affected by RC addition ($P>0.05$).

No dose response of RC on hexanal was observed based on the absence of a significant difference between the 5RC and 30RC treatments. Unexpectedly, at days 1 and 20 of chilled storage heptanal and octanal were detected in the 30RC samples, while these volatiles were not found in the 5RC samples.

For all treatments, hexanal values increased with time of chilled storage (P<0.01). However, this increase was more intense in the NC-treated samples than in the 5RC-, 30RC and PC-treated samples. Heptanal increased with time (P<0.001) in the NC samples and was not detected in the frankfurters containing 5 g/kg RC at day 1 and 20. For the 30RC and PC samples, heptanal values were higher (P<0.05) at day 40 compared to the other days of storage. Octanal increased in NC, 5RC and PC with time (all P<0.01), while in the 30RC-samples octanal did not change with time of storage (P>0.05). Nonanal increased (P<0.05) in the NC samples, while for the other treatments no changes were observed (all P>0.05).

Table 1.1. Lipid oxidation (AAU $\times 10^6$) during chilled storage of frankfurters with added dog rose extract

		NC	5RC	30RC	PC	*SEM[1]*	*P*
Hexanal	**day 1**	1.35^{c,x}	0.70^{c,xy}	1.50^{b,x}	0.30^{b,y}	*0.15*	*0.004*
	20	16.35^{b,x}	1.60^{bc,y}	1.30^{b,y}	0.60^{a,y}	*1.75*	*<0.001*
	40	23.15^{ab,x}	2.36^{b,y}	2.50^{b,y}	0.48^{ab,y}	*2.43*	*<0.001*
	60	27.52^{a,x}	7.08^{a,y}	4.46^{a,yz}	0.53^{a,z}	*2.76*	*<0.001*
	SEM[2]	*2.68*	*0.66*	*0.38*	*0.04*		
	P	*<0.001*	*<0.001*	*<0.001*	*0.011*		
Heptanal	**day 1**	0.20^b	n.d.	0.18^b	0.10^b	*0.02*	*0.149*
	20	0.55^{a,x}	n.d.	0.32^{b,y}	0.10^{b,y}	*0.06*	*<0.001*
	40	0.63^{a,xy}	0.32^y	0.98^{a,x}	0.30^{a,y}	*0.08*	*<0.001*
	60	0.86^{a,x}	0.45^y	0.39^{b,yz}	0.17^{b,z}	*0.07*	*<0.001*
	SEM[2]	*0.07*	*0.05*	*0.09*	*0.02*		
	P	*<0.001*	*0.216*	*<0.001*	*<0.001*		
Octanal	**Day 1**	0.30^{b,x}	n.d.	0.20^{xy}	0.10^{b,y}	*0.03*	*0.019*
	20	0.60^{b,x}	n.d.	0.20^y	0.40^{ab,xy}	*0.06*	*0.006*
	40	0.70^{ab,x}	0.22^{b,y}	0.40^{xy}	0.74^{a,x}	*0.07*	*0.005*
	60	1.10^{a,x}	0.78^{a,xy}	0.50^y	0.78^{a,xy}	*0.08*	*0.045*
	SEM[2]	*0.09*	*0.08*	*0.05*	*0.08*		
	P	*0.004*	*<0.001*	*0.092*	*<0.001*		
Nonanal	**day 1**	1.60^b	0.90	1.90	1.15	*0.16*	*0.107*
	20	2.95^{ab}	1.70	1.80	1.40	*0.24*	*0.083*
	40	3.08^{ab}	1.84	2.16	2.58	*0.23*	*0.270*
	60	5.02^a	2.48	2.05	2.75	*0.44*	*0.051*
	SEM[2]	*0.41*	*0.24*	*0.22*	*0.27*		
	P	*0.010*	*0.138*	*0.951*	*0.054*		

5RC: 5 g/kg *Rosa canina* L.*;*30RC: 30 g/kg *Rosa canina* L.; PC: Positive control, NC: Negative control;

[a–c] Effect of storage: values with a different letter within a column of the same treatment are different (P < 0.05);

[x-z] Effect of treatment: values with a different letter within a row of the same storage day are different (P < 0.05);

[1]Standard error of the mean within the same storage day (n=16);

[2]Standard error of the mean within the same treatment (n=16).

Similarly to lipid oxidation, protein oxidation occurred scarcely in the PC frankfurters, while the highest amounts of protein oxidation products were found in the NC samples (Table 1.2). Intermediate values for the RC treatments and no significant differences (all P>0.05) between the two RC doses were observed for both AAS and GGS. The protein oxidation products increased for all treatments during the chilled storage period (all P<0.001). Overall, higher amounts of AAS were formed during the experiment compared to the amounts of GGS. Both protein carbonyls, AAS and GGS, increased more intensively during 60 days of storage in the NC-frankfurters, followed by the 5RC, 30RC and finally the PC-samples. It is worth noting that GGS was only formed after 20 days of chilled storage, except for the NC frankfurters, while AAS was present from day 1 on.

Table 1.2 Protein oxidation (nmol carbonyls/mg protein) during chilled storage of frankfurters with added dog rose extract

		NC	5RC	30RC	PC	*SEM[1]*	*P*
AAS	**day 1**	0.72[b,x]	0.35[c,y]	0.36[b,y]	0.14[c,z]	*0.06*	*<0.001*
	20	1.03[b,x]	0.76[bc,xy]	0.55[b,yz]	0.18[bc,z]	*0.09*	*<0.001*
	40	1.26[b,x]	1.06[ab,x]	1.00[a,x]	0.27[ab,y]	*0.10*	*<0.001*
	60	2.02[a,x]	1.53[a,xy]	1.12[a,y]	0.38[a,z]	*0.17*	*<0.001*
	SEM[2]	*0.14*	*0.13*	*0.08*	*0.03*		
	P	*<0.001*	*<0.001*	*<0.001*	*<0.001*		
GGS	**day 1**	0.18[c]	n.d.	n.d.	n.d.	*0.02*	*-*
	20	0.41[b]	n.d.	n.d.	n.d.	*0.06*	*-*
	40	0.60[b,x]	0.20[b,y]	0.14[b,y]	0.22[y]	*0.05*	*<0.001*
	60	0.90[a,x]	0.42[a,y]	0.37[a,yz]	0.18[z]	*0.07*	*<0.001*
	SEM[2]	*0.07*	*0.05*	*0.04*	*0.03*		
	P	*<0.001*	*0.021*	*<0.001*	*0.111*		

5RC: 5 g/kg *Rosa canina* L.*;* 30RC: 30 g/kg *Rosa canina* L.; PC: Positive control, NC: Negative control;,AAS: α-amino adipic semialdehyde, GGS: γ-glutamic semialdehyde;
[a–c] Effect of storage: values with a different letter within a column of the same treatment are different (P < 0.05);
[x-z] Effect of treatment: values with a different letter within a row of the same storage day are different (P < 0.05);
[1]Standard error of the mean within the same storage day (n=16);
[2]Standard error of the mean within the same treatment (n=16).

Addition of RC extracts had a significant effect on the colour parameters of the frankfurters (Table 1.3). Dose-dependent responses of RC (P<0.05) were observed as lower L* values and higher a* and b* values were found on the 30RC frankfurters compared to those containing 5 g/kg RC. Throughout the whole storage period, the a* values of the RC frankfurters were higher (P<0.05) compared to the NC samples and the a* values of the PC frankfurters were higher (P<0.05) compared to the other groups.

During the storage period, L* values of the 5RC, 30RC and PC samples decreased slightly (all P<0.05), in contrast to the NC frankfurters which did not change (P>0.05) during the storage period. The a* values of the 5RC and NC frankfurters did not change during storage (both P>0.05), while an increase was observed for the 30RC and PC samples (both P<0.01), however the difference was less than 1 and 2 units respectively. The b* values of the 5RC, 30RC and NC frankfurters increased during storage (all P<0.05), however all these differences were also small. No clear pattern could be found for the b* values of the PC frankfurters. Also the internal surface of the frankfurters was measured (data not shown). In general the lightness of the frankfurters was similar compared to the external surface, while the redness was lower and the yellowness higher on the outside of the frankfurters compared to the internal surfaces. Similar to the external surface, higher a* values and b* values were found in the RC frankfurters compared to NC, but the highest a* values were found in the PC frankfurters.

Table 1.3. Colour parameters of the external surfaces of frankfurters with added dog rose extract during chilled storage

		NC	5RC	30RC	PC	*SEM[1]*	*P*
L*	**Day 1**	75.40[w]	75.88[a,w]	74.03[a,x]	76.37[a,w]	*0.24*	*<0.001*
	20	75.83[w]	74.78[ab,wx]	73.79[a,x]	73.82[b,x]	*0.23*	*<0.001*
	40	76.10[w]	74.65[b,x]	72.51[b,y]	74.23[b,x]	*0.31*	*<0.001*
	60	76.16[w]	74.18[b,x]	72.20[b,y]	73.13[b,xy]	*0.36*	*<0.001*
	SEM[2]	*0.22*	*0.19*	*0.23*	*0.29*		
	P	*0.642*	*0.006*	*<0.001*	*<0.001*		
a*	**Day 1**	3.83[y]	4.21[y]	5.08[b,x]	7.99[c,w]	*0.38*	*<0.001*
	20	3.61[z]	4.09[y]	5.20[b,y]	9.09[b,w]	*0.50*	*<0.001*
	40	3.89[z]	4.40[y]	5.80[a,x]	9.59[ab,w]	*0.47*	*<0.001*
	60	3.70[z]	4.26[y]	5.18[b,x]	9.83[a,w]	*0.51*	*<0.001*
	SEM[2]	*0.05*	*0.05*	*0.09*	*0.16*		
	P	*0.151*	*0.107*	*0.008*	*<0.001*		
b*	**Day 1**	11.01[b,y]	12.20[b,x]	15.50[b,w]	10.58[a,y]	*0.45*	*<0.001*
	20	11.17[b,y]	12.16[b,x]	15.64[b,w]	8.67[b,z]	*0.58*	*<0.001*
	40	13.00[a,x]	12.71[a,x]	15.44[b,w]	9.20[b,y]	*0.48*	*<0.001*
	60	12.40[a,x]	12.80[a,x]	17.53[a,w]	10.31[a,y]	*0.57*	*<0.001*
	SEM[2]	*0.21*	*0.10*	*0.23*	*0.19*		
	P	*<0.001*	*0.027*	*<0.001*	*<0.001*		

5RC: 5 g/kg *Rosa canina* L.*;* 30RC: 30 g/kg *Rosa canina* L.; PC: Positive control, NC: Negative control;

[a–c] Effect of storage: values with a different letter within a column of the same treatment are different (P < 0.05);

[w–z] Effect of treatment: values with a different letter within a row of the same storage day are different (P < 0.05);

[1] Standard error of the mean within the same storage day (n=20);

[2] Standard error of the mean within the same treatment (n=20).

The addition of RC extracts resulted in several significant changes on the texture parameters of the frankfurters (Table 1.4). However, some differences were very small and no obvious trends could be found. Therefore, data concerning springiness, cohesiveness and resilience are not shown. In general, but with some exceptions, the 30RC treatment led to frankfurters with higher hardness and related chewiness values, compared to the other treatments. No differences in hardness and chewiness were found between the 5RC and PC samples (P>0.05), except for day 40 with higher values in the former (P<0.05). The adhesiveness was higher in the NC samples compared to the 30RC samples (P<0.05) at more than 20 days of storage.

Throughout the storage period changes were observed for the texture parameters. When comparing the results at the end of the experiment with the start of the experiment, the texture parameters were affected the most in the NC frankfurters: the hardness and chewiness decreased (P<0.05) and the adhesiveness increased (P<0.05). On the other hand no changes (P>0.05) between day 1 and day 60 were found in the 5RC and commercial frankfurters (PC) for hardness, chewiness and adhesiveness For the 30RC treatment decreased adhesiveness values were found (P<0.05) along the storage period, while hardness and chewiness were not affected during the storage period (both P>0.05).

Table 1.4. Texture parameters measured during chilled storage of frankfurters with added dog rose extract

		NC	5RC	30RC	PC	*SEM[1]*	*P*
Hardness	**day 1**	3.63[a,x]	3.16[b,y]	3.44[xy]	3.26[ab,xy]	*0.06*	*0.045*
($10^3 \times$g)	**20**	2.85[b,y]	3.06[b,y]	3.50[x]	2.87[b,y]	*0.06*	*<0.001*
	40	2.89[b,y]	3.86[a,x]	3.88[x]	3.17[ab,y]	*0.10*	*<0.001*
	60	2.82[b,y]	2.95[b,y]	3.71[x]	3.38[a,xy]	*0.11*	*0.007*
	SEM[2]	*0.09*	*0.08*	*0.06*	*0.07*		
	P	*0.003*	*<0.001*	*0.056*	*0.047*		
Chewiness	**day 1**	2.04[a]	1.92[b]	2.07	1.96	*0.03*	*0.329*
($10^3 \times$g)	**20**	1.73[ab,y]	1.82[b,y]	2.10[x]	1.74[y]	*0.03*	*<0.001*
	40	1.68[b,y]	2.28[a,x]	2.30[x]	1.87[y]	*0.06*	*<0.001*
	60	1.57[b,y]	1.68[b,y]	2.18[x]	1.97[xy]	*0.07*	*0.003*
	SEM[2]	*0.05*	*0.05*	*0.04*	*0.04*		
	P	*0.009*	*<0.001*	*0.098*	*0.085*		
Adhesiveness	**day 1**	-28.93[b]	-27.80[ab]	-16.49[a]	-19.02	*1.96*	*0.050*
(g$\times$s)	**20**	-12.76[a,x]	-31.12[b,y]	-32.39[b,y]	-22.31[xy]	*2.16*	*<0.001*
	40	-13.89[a,x]	-17.93[a,xy]	-28.36[ab,y]	-17.85[xy]	*1.97*	*0.045*
	60	-16.82[ab,x]	-15.60[a,x]	-32.94[b,y]	-18.76[xy]	*2.11*	*0.011*
	SEM[2]	*2.05*	*1.91*	*2.16*	*1.83*		
	P	*0.023*	*0.004*	*0.012*	*0.841*		

5RC: 5 g/kg *Rosa canina* L.*;* 30RC: 30 g/kg *Rosa canina* L.; PC: Positive control, NC: Negative control;

[a–b] Effect of storage: values with a different letter within a column of the same treatment are different (P < 0.05);

[x-y] Effect of treatment: values with a different letter within a row of the same storage day are different (P < 0.05);

[1] Standard error of the mean within the same storage day (n=36);

[2] Standard error of the mean within the same treatment (n=36).

DISCUSSION

According to the literature, the ascorbic acid content of dog rose ranges between 23.7-27.5 g/kg fresh matter (Demir & Ízcan, 2001; Egea et al., 2010). Assuming that the major part of ascorbic acid can be extracted from the fresh matter, about 0.125 and 0.750 g ascorbic acid per kg frankfurter could be present in the 5RC or 30RC frankfurters, respectively. These amounts are comparable to what is usually added in commercial frankfurters as sodium ascorbate. For the total phenolic content of the frankfurters, approximately 200 and 1000 mg GAE/kg for 5RC and 30RC respectively could be expected, taking into account the measured total phenolic content of the RC extracts. Although these values are only indicative and not supported by analyses on the material used in the present study, they provide insight on the antioxidant potential of RC in the experimental frankfurters. According to the results, the incorporation of RC in frankfurters inhibited both lipid and protein oxidation. In previous studies, specific phenolic components of dog rose such as cyanidins and catechins were found to be efficient inhibitors of lipid and protein oxidation in emulsions and suspensions of meat proteins (Estévez et al., 2008; Estévez & Heinonen, 2010). The present study confirms the efficiency of dog rose phenolics as antioxidants in a more complex food system. The previously mentioned authors attributed the antioxidant effects on proteins to the ability of dog rose phenolics to act as radical scavengers and metal chelators. These mechanisms are also applicable to the present study as protein carbonylation is usually initiated by reactive oxygen species (ROS) and involves metal-catalyzed oxidation of particular amino acid residues (Estévez, 2011). This outcome compares well with the effects previously reported in cooked burger patties (Ganhão et al., 2010c; Ganhão et al., 2010c). It is worth noting that the RC extracts are as efficient as the combination of sodium nitrite and sodium ascorbate (PC samples) for lipid oxidation, while the latter was more effective against protein oxidation. Likewise, previous studies that investigated the effect of various antioxidants on the oxidative stability of meat products reported lower efficacy of such antioxidant

compounds against protein oxidation than against lipid oxidation (Mercier et al., 2004; Estévez et al., 2008; Haak et al., 2009). Estévez et al. (2008) also found that phenolic compounds were more effective against lipid oxidation than in preventing protein oxidation *in vitro*. These authors suggested that the phenolic compounds could be mainly located in the inner layer of the interphase, exposed to the lipid phase where lipid oxidation occurs. In addition, the likely covalent binding between phenolic compounds and proteins was suggested to decrease the ability of the former to act as a radical scavenger. This implies that the direct antioxidant effect of phenolic compounds on protein oxidation may be rather limited. However, protein oxidation could be indirectly inhibited by phenolic compounds by decreasing the formation of primary lipid oxidation products that are known to initiate protein oxidation (Estévez, 2011). In addition, ascorbic acid and nitrite have been known for a long time to exhibit antioxidant activity against lipid oxidation (Honikel, 2008; Ranken, 1981), while this has been less clear for protein oxidation. Different authors reported both pro- and antioxidant activity of ascorbic acid against carbonyl formation (Estévez, 2011) and according to Vossen et al. (2012a), no effect of nitrite on carbonyl formation was found in liver pâté. In the present study, the combination of those additives shows efficient protection against the formation of specific protein carbonyls in frankfurters.

Irrespective of the treatment or storage day, higher amounts of AAS were formed during oxidation compared to GGS. Moreover, at the onset of the trial, AAS was present in all samples, while GGS lacked in some cases. AAS is derived from lysine, while GGS is the main oxidation product originating from arginine and proline (Requena et al., 2001). Apparently, in this study lysine was more easily oxidized compared to proline and arginine, which was also found by Utrera et al. (2011). Nevertheless, opposite results were published by Armenteros et al. (2009) and Ganhão et al. (2010c), in which higher amounts of GGS and lower amounts of AAS were found in different meat products. Protein carbonylation has been linked to loss of protein

functionality and deterioration of various sensory and technological properties of muscle foods, including its water holding capacity (Estévez et al., 2011; Lund et al., 2011). The inhibition of protein carbonylation by dog rose extracts could provide a substantial benefit to the quality characteristics of the present frankfurters. In addition, oxidative modifications of proteins can lead to the loss of essential amino acids and a decreased digestibility affecting ultimately the nutritional quality of muscle foods (Lund et al., 2011). The oxidation of proteins may cause an altered susceptibility of protein substrates to proteolytic enzymes, as the formation of protein aggregates and the oxidative degradation of specific amino acid side chains could alter recognition sites both chemically and physically leading to a decreased proteolytic susceptibility (Estévez 2011). This implies that even small amounts of oxidative modifications could have an effect on the nutritional value.

Regarding the lipid oxidation products, hexanal was more abundantly present compared to the other volatiles. Furthermore, during storage of the frankfurters, hexanal increased more intensively compared to the other volatiles. In literature, hexanal is reported to be the most sensitive indicator for lipid oxidation (Ahn et al., 1998). However, other lipid-derived volatiles such as heptanal, octanal, and nonanal should also be taken into account due to their low flavour threshold values (Specht & Baltes, 1994). Hexanal and heptanal are degradation products from long chain polyunsaturated n-6 fatty acids, mainly linoleic acid, while nonanal, octanal as well as heptanal arise from oxidation of monounsaturated n-9 fatty acids, e.g. from oleic acid (Meynier et al., 1998). Long chain polyunsaturated fatty acids are known to be less stable towards oxidation compared to monounsaturated fatty acids, which in the present study resulted in high hexanal values.

In general, no dose response of RC on lipid and protein oxidation was observed. Moreover, in some cases higher amounts of lipid derived volatiles were measured in the 30RC samples, implying that the addition of natural antioxidants should be applied with care. As

aforementioned, the antioxidant activity of phenolic compounds is attributed to their free radical scavenging and metal chelating activities, in which hydroxyl groups attached to phenolic rings play an important role (Bravo, 1998). However, during auto-oxidation of these antioxidants, hydroxyl forms can convert to their corresponding pro-oxidant quinone structures. The overall pro- or antioxidant effect displayed by plant phenolics might therefore be the result of the balance between both forms (Estévez & Heinonen, 2010). Moreover, the effect of a certain potential antioxidant might vary considerably depending on a complex interaction between various factors, involving the type and concentration of active compound(s) and the nature of the food system (Madsen & Bertelsen, 1995). In addition, also ascorbic acid may act as a pro-oxidant in specific conditions, most likely due to the strong reducing power and weak metal-chelating ability (Yen et al., 2002). Various other studies have reported pro-oxidant effects of phenolic compounds or ascorbic acid, both for lipid or protein oxidation in pork (Haak et al., 2009), frankfurters (Estévez et al., 2007b) and chicken (Tang et al, 2000). However, although in some cases 5RC was more effective against lipid and protein oxidation compared to 30RC, both RC treatments showed clear antioxidant activities compared to the negative control without antioxidants.

For the colour, only the PC treated frankfurters showed the common pink colour found in commercial frankfurters. Sensory analysis should be carried out to investigate the consumer's acceptability towards these experimental frankfurters. Noteworthy are the higher a* values in the RC frankfurters compared to the NC samples: perhaps some nitrosopigments were formed, as RC contains approximately 1 g nitrate per kg fresh matter (Cakilcioglu & Khatun, 2011). Taking into account this amount, about 5 and 30 mg nitrate per kg frankfurter could roughly be present in the 5RC and 30RC frankfurters respectively. However, nitrate is only effective after being reduced to nitrite, which can be accomplished by microorganisms found in the natural flora of meat (Sindelar et al., 2007b). Although this reduction is only possible in raw batters and not in

cooked meat products, no more than 4 to 6 mg nitrite/kg frankfurters is necessary for cured colour development in frankfurters (Fox Jr (1987), as cited in Heaton et al. (2000)). These small amounts could possibly have been formed during the manufacturing of the frankfurters. It would thus be of interest to study the potential of RC in colour formation in more detail. Another possible explanation could be that the extracts, which were slightly coloured, contributed to the higher a* values. The a*values of NC and 5RC remained stable during storage, but what is unusual about the results of the PC and 30RC treatments, is that the a* values increased during storage, while a loss in redness was expected. This increase is difficult to explain as various endogenous factors can change the conditions of the meat, such as pH, reducing conditions, degree of denaturation, and reactivity of endogenous meat compounds, which can affect the chemical state, structure, and reactivity of the pigments. Some of these factors may result in pinking of cooked meat (Holownia et al., 2011). The b* value of the RC frankfurters were higher compared to the other treatments and a dose response was observed. As the extracts were yellowish, this result could be expected.

Texture is a major parameter of cooked sausages and consumer acceptance of food products strongly depends on textural characteristics. The mechanical characteristics hardness, chewiness and adhesiveness can be explained in terms closely related to actual consumer perception: hardness is the force required to compress a substance between the molar teeth, chewiness is the length of time required to masticate a sample at a constant rate of force application, to reduce it to a consistency suitable for swallowing and adhesiveness is the force required to remove material that adheres to the mouth during the normal eating process (Bourne, 1982). When changing the composition of a well known meat product such as frankfurters, one should verify that the desired textural characteristics are maintained. In this study, the effect of omitting sodium nitrite as well as the effect of RC addition in the frankfurters should be explored. In a work carried out by Dong et al. (2007), altering the sodium nitrite concentrations from 0 to 150

mg/kg, resulted in changed texture attributes and the nitrite concentration was negatively correlated with hardness and adhesiveness. In this study, no differences in texture parameters was found between the nitrite containing (PC) and nitrite-free (NC) frankfurters, which is in contrast with the results found by Dong et al. (2007). On the other hand, adding different doses of RC did affect the texture parameters dose-dependently. On average, frankfurters containing 30RC were harder and showed an increased chewiness compared to 5RC, NC and PC. Ganhão et al. (2010b) found an increased hardness in cooked pork patties containing 30 g/kg RC fruits compared to a control without added fruit extracts, while no significant effects on other texture parameters such as adhesiveness and chewiness were found. In previous studies, the deterioration of particular texture traits such as hardness during storage of meat products was closely linked to the oxidative deterioration of meat proteins (frankfurters, Estévez et al., 2005a and Estévez et al., 2011; cooked patties, Ganhão et al., 2010b). On the same line, the impact of phenolic-rich extracts on the texture properties was ascribed to the ability of these phytochemicals to inhibit protein oxidation and hence, the derived texture deterioration. The connection between protein oxidation changes and texture deterioration during chilled storage of frankfurters was not observed in the present study.

In addition to the important function of nitrite regarding colour and oxidative stability of the meat product, nitrite plays a key role in cured meat as a bacteriostatic and bacteriocidal agent. Nitrite is a strong inhibitor of anaerobic bacteria, most importantly *Clostridium botulinum* and contributes to control of other micro-organisms such as *Listeria monocytogenes* (Sebranek & Bacus, 2007). Therefore, caution should be exercised when meat products without or low amounts of nitrite are produced. Although not investigated in this research, it should be explored if RC can be used against harmful micro-organisms. From literature it appears that RC has antibacterial activities (Kumarasamy et al., 2003) and other polyphenolic-rich fruits were reported to be effective against human pathogens such as *Clostridium* (Heinonen, 2007).

CONCLUSIONS

Addition of *Rosa canina* L. revealed clear protection against lipid and protein oxidation in frankfurters during 60 days of chilled storage, while the textural properties of the frankfurters only changed slightly. Data also suggest that *Rosa canina* L. extracts may have the potential to contribute to pink colour formation. Further studies should include an assessment of microbiological risk and sensory research on the acceptability of these frankfurters to verify the efficacy of *Rosa canina* L. to extend the shelf life of sodium nitrite-free frankfurters without compromising the safety of the meat product.

ACKNOWLEDGEMENTS

The antioxidant role of nitrite is partly replaced by a plant extract containing ascorbic acid (CH1).

Can sodium ascorbate replace the antioxidant role of sodium nitrite in liver pâté?

CHAPTER 2

EFFECT OF SODIUM ASCORBATE DOSE ON THE SHELF LIFE STABILITY OF REDUCED NITRITE LIVER PÂTÉS

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ABSTRACT

The effect of sodium ascorbate (SA; 500, 750, 1000 mg/kg) and sodium nitrite (SN; 40, 80, 120 mg/kg) dose on the shelf-life stability of liver pâtés was investigated in a full factorial design. Clear dose-dependent responses of the added SN or SA were found for the concentrations of nitrite, ascorbic acid and dehydroascorbic acid in the raw batters and in the cooked pâtés before and after 48h of chilled display. Decreasing the SN dose to 80 mg/kg had no negative impact on the colour stability (a* value) and lipid oxidation (TBARS), and no additional antioxidant effect of SA was noticed. Lowering SN to 40 mg/kg resulted in proper colour formation, but the colour stability was inferior and lipid oxidation increased. Yet, increasing the amount of SA, at this low SN dose, resulted in lower TBARS values. Decreasing the SN dose to 80 or 40 mg/kg had no distinct effect on protein oxidation, which was however only measured by carbonyl content.

INTRODUCTION

Liver pâté, a traditionally cooked and widely consumed meat product in many countries, consists of a comminuted mixture of liver and fat to which additives are added. Due to its chemical composition and manufacturing process, liver pâté is considered as being a product highly susceptible to oxidation (Estévez et al., 2007a). Liver pâté is rich in fat and non-haem iron, with the latter being considered as the most important pro-oxidant in meat systems (Kanner, 1994). In addition, mincing and cooking makes meat products more susceptible to oxidation compared to fresh meat, due to the facilitated interaction between free fatty acids and oxygen in the presence of catalysts such as heat and metalloproteins (Morrissey et al., 1998). Oxidation leads to several changes in fat components and meat pigments, thereby reducing the quality of the product in terms of taste, colour and shelf-life. In the past, the main focus on oxidation in meat and meat products was on colour and lipid oxidation, but nowadays also protein oxidation seems to influence specific meat quality traits (Lund et al., 2011).

Two important ingredients influencing the oxidative stability of liver pâté are nitrite and ascorbic acid (AA). Nitrite plays an important role during meat processing, colour development, lipid oxidation, flavour formation and microbiological safety (Honikel, 2008). After adding nitrite to a batter of meat, the nitrite has different fates: it is partially oxidized to nitrate by sequestering oxygen, bound to myoglobin and bound to proteins or other substances (Honikel, 2008). The rate of nitrite depletion is dependent on different factors such as pH, initial nitrite concentration, processing technique and storage temperatures, meat-to-water ratio and the presence of antioxidants (Kilic et al., 2002). According to Honikel (2008) residual nitrite levels in meat products vary between 5 and 20% of the ingoing amount. The antioxidant AA works as a potent radical-scavenging component, improving the oxidative stability and colour formation of meat and meat products (Perlo et al., 1995; Sahoo & Anjaneyulu, 1997). Also, AA can act synergistically with tocopherols by regenerating and restoring their antioxidant properties (Niki

et al., 1995) and interacts with nitrite (Izumi et al., 1989). When nitrite is added to a batter, an equilibrium reaction occurs between nitrite and nitrous acid. From the nitrous acid, nitric oxide can be formed by the action of endogenous meat enzymes or reducing agents (Ranken, 1981). For the colour formation, nitric oxide reacts almost instantly with metmyoglobin forming nitrosylmetmyoglobin. The nitrosylmetmyoglobin is subsequently reduced to the red cured colour nitrosylmyoglobin. As AA is a strongly reducing agent, it plays an important role in the colour formation of cured meat products since it accelerates the reducing steps (Ranken, 1981).

The American Institute for Cancer Research (2007) suggests that dietary nitrites are to be considered as human carcinogens, because they may be converted to carcinogenic N-nitroso compounds (Demeyer et al., 2008). Consumers are concerned about the possible harmful effects of nitrite and the meat industry is challenged to reduce the concentrations of nitrite in their meat products. Regarding the formation of N-nitroso compounds, knowledge about the residual nitrite concentration could be just as important as focusing on the initially added nitrite concentration.

Because of the significant role of AA in the colour development and oxidative stability of meat products and the concerns about residual nitrite, a partial replacement of nitrite with AA in meat processing could be valuable. Generally, an amount of 40 mg/kg ingoing nitrite is considered to be sufficient for colour-fixing purposes and to achieve the expected cured meat appearance (USDA, 1995). The colour stability and oxidative stability at this low dose is however less documented, especially in liver pâté. Therefore, the objective of this study is to investigate the effect of increased sodium ascorbate supplementation on the residual nitrite levels, colour stability, lipid and protein oxidation as well as antioxidant concentrations of reduced nitrite liver pâtés.

MATERIALS AND METHODS

1. Experimental set-up and sampling

The experiment consisted of a 3×3 full factorial design with three levels of sodium nitrite (SN, E250) (Kerry Ingredients and Flavours, Bornem, Belgium) (40, 80 and 120 mg/kg) combined with three levels of sodium ascorbate (SA, E301) (Kerry Ingredients and Flavours, Bornem, Belgium) (500, 750 and 1000 mg/kg) added to the batters. In commercial conditions, 120 mg/kg SN and 500 mg/kg SA are generally used (according to the information given by the supplier). The basic composition of each batch was based on a commercial recipe (g/kg): 290 g pork liver, 380 g pork subcutaneous fat, 290 g broth (the boiling water in which the fat was cooked), 18.0 g sodium chloride, 5.0 g dextrose, 10.0 g sodium caseinate and spices (2.0 g white pepper, 0.5 g nutmeg, 0.5 g ginger, 0.2 g cardamom, 0.5 g onion powder). All spices were purchased from RAPS (Beringen, Belgium) and the other additives were from Kerry Ingredients and Flavours (Bornem, Belgium).

The preparation of the batters and cooking of the pâtés was performed on three subsequent days, with the preparations for all levels of SA and per SN dose done on one day. Beforehand, separate mixtures of raw livers and subcutaneous fat from several commercial slaughter pigs (Impens NV, Melle, Belgium) were made. These mixtures were divided in three batches to be used for the three processing days, frozen until -21 °C and stored for maximum three days. For the preparation of the batter, first the cold liver part was minced for 8 minutes at 3000 rpm (Stephan vertical cutter-mixer, model UM12-F/3, consisting of two blades aligned at 180 degrees to each other), curing salts (sodium nitrite and sodium chloride) were added and the cutting process was continued under vacuum for 2 minutes at 1500 rpm. The cured liver was kept refrigerated (1-7 °C) during the preparation of the fat. The fat was scalded for 20 minutes in boiling water until it reached a temperature of 40 °C. The fat was then minced and homogenised with sodium caseinate and broth for 5 minutes at 51 °C in the Stephan vertical cutter-mixer. Subsequently the

cured liver and the other additives were added to this warm emulsion in the cutter. The mixture was further homogenised for 3 minutes until a homogeneous raw batter of 40 °C was obtained. Finally, the ready batters were manually distributed into metal cans until completely full (250 g, can height: 6 cm) and these were then hermetically closed using a can sealing machine (Indosa, type M160).

Three cans per treatment were immediately frozen (-21 °C) for further analysis, and are referred to as the batter samples. The other cans (six per treatment) were cooked in saturated steam conditions at 75 °C for 90 minutes. The cans were rapidly cooled in an ice bath and were stored in the dark at 4 °C. After 7 days, the cans were opened and two slices of 2 cm thickness were sampled after removing 1 cm of the top and bottom layer. Six slices per treatment were immediately vacuum packed and stored at -21 °C for further analysis. Six other slices per treatment were wrapped in an oxygen permeable polyethylene film (purchased from a local supermarket (Delhaize), thickness 0.010 mm), and placed in an illuminated chilled cabinet (1000 lux, 4 °C). Permeability characteristics of the film were not available from the supplier, but according to Massey (2002), this kind of low density polyethylene film has an oxygen gas permeability in the range of 255-470 $cm^3 \times mm \times m^{-2} \times 24h^{-1} \times atm^{-1}$ and a water vapour transmission rate in the range of 1.25-1.85 $g \times mm \times m^{-2} \times 24h^{-1}$. After 48 h of chilled display, the polyethylene film was removed and the samples were vacuum packed and stored at -21 °C until further analysis. All analyses were performed in duplicate.

2. Composition analyses

Dry matter, crude protein and crude fat content were analysed on three pooled pâté samples according to the ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods, respectively. The pH was measured on minced samples using a glass pH-electrode.

The AA and dehydroascorbic acid (DHAA) content was determined according to the method of Zapata & Dufour (1992) and Dodson et al. (1992). This assay is based on the reaction of DHAA with orthophenylenediamine (OPD). Briefly, AA and DHAA were extracted using methanol/water (5/95; v/v) containing 0.1 M citric acid and 0.2 mM EDTA. DHAA is able to react with OPD, but AA first needs to be converted into DHAA, using active carbon. By measuring total DHAA (i.e. the present DHAA and DHAA formed from converted AA), and DHAA present in the samples, the AA concentration was calculated. Samples were analysed by reversed phase HPLC (Agilent, Waldbronn, Germany), using a Pursuit XRS C18 column (15 cm $\times$ 4.6 mm $\times$ 5 µm; Varian, Sint-Katelijne-Waver, Belgium) with fluorimetric detection at an excitation and emission wavelength of 350 and 430 nm respectively. The mobile phase was a mixture of methanol/water (5/95; v/v), containing 5 mM cetrimide and 50 mM KH_2PO_4 (pH 4.6). The elution was performed at a flow rate of 1.0 ml/min. Quantification was done by comparison of peak areas with those obtained from a standard solution of converted (L)-ascorbic acid. Results were expressed as mg AA or DHAA/kg batter or pâté.

The residual nitrite was determined by the ISO 2918-1975 reference method. After a reaction with sulfanilamide and naftylethylenediamine, nitrite was measured spectrophotometrically at 538 nm. The nitrite concentration was calculated based on a standard curve obtained with SN and expressed as mg/kg batter or pâté.

The α-tocopherol content was determined according to the method of Desai (1984) with some improvements. After saponification and n-hexane extraction, all samples were analysed by reversed phase HPLC (GE Healthcare, Diegem, Belgium), using a Supelcosil LC18 column (25 cm $\times$ 4.6 mm $\times$ 5 µm; Sigma-Aldrich, Bornem, Belgium). The mobile phase was a mixture of methanol/water (97/3; v/v) and the elution was performed at a flow rate of 2.0 ml/min. UV-detection was accomplished at a wavelength of 292 nm. The α-tocopherol content of the samples

was determined by comparison of peak areas with those obtained from a standard curve of α-tocopherol. The results were expressed as mg α-tocopherol/kg batter or pâté.

3. Oxidative stability measurements

Lipid oxidation was assessed by measuring the 2-thiobarbituric acid-reactive substances (TBARS), with the extraction method using perchloric acid (0.64 M) as described by Ventanas et al. (2006). In this method, malondialdehyde (MDA), a secondary oxidation product, forms a coloured complex with 2-thiobarbituric acid (TBA). This complex was determined spectrophotometrically at 532 nm. Results were expressed as mg MDA/kg batter or pâté.

Protein oxidation was assessed by determining the carbonyl content of the samples. The protein were extracted from the meat matrix with of phosphate buffer (20 mM, pH 6.5 containing 0.6M NaCl) and four aliquots of the homogenate were treated with TCA (10% w/v) to precipitate the proteins. The measurement of protein carbonyls following their covalent reaction with 2,4-dinitrophenylhydrazine (DNPH) was done according to Ganhão et al. (2010b). This reaction leads to the formation of a stable 2.4-dinitrophenyl hydrazone product. Total carbonyl content was quantified spectrophotometrically at 370 nm, using a molar absorption coefficient of 21.0/(mM·cm) and expressed as nmol DNPH incorporated/mg protein. Since the pâtés were manufactured from the same homogeneous mixtures of livers and fat, the variation in the composition of the liver pâtés was minimal. Therefore, to calculate the carbonyl content, the average protein content obtained from the crude protein analysis (8.56 g/100g pâté) was used.

Colour coordinates (CIE L*a*b* colour system 1976) were measured with a HunterLab Miniscan Minolta XE plus spectrocolorimeter (light source of D65, standard observer of 10°, 45°/0° geometry, 1 inch. light surface, white standard). Samples were measured in six fold, every 10 minutes for the first 3 h, every hour from 3 h until 8 h and at 24 h and 48 h of display in the

chilled cabinet. The colour fading, measured as a decline in a* values, was fitted to a non-linear, two-phase exponential decay curve (GraphPad Prism5, Demo2010) using the equation:

$$Y = \text{plateau} + A \times \exp(-K\text{fast} \times X) + B \times \exp(-K\text{slow} \times X)$$

with $A = (Y0-\text{plateau}) \times \%\text{Fast} \times 0.01$

$B = (Y0-\text{plateau}) \times (100-\%\text{Fast}) \times 0.01$

Kfast and Kslow (expressed in minutes^{-1}) = the two rate constants

Y0 = initial a* value (intercept)

Plateau = the ultimate a* value after 48 h of display

%Fast = the fraction of the span (from Y0 to plateau) accounted for by the faster of the two components.
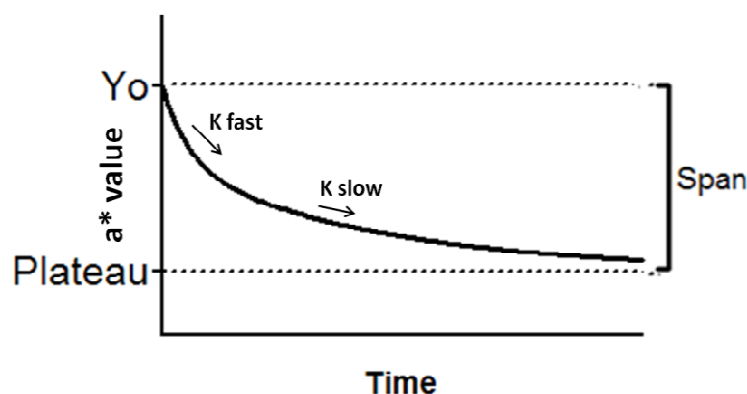
Figure 2.1. Illustration of a two-phase exponential decay curve

Six replicate pâté samples per treatment were measured and fitted separately, and values for Y0, plateau, %Fast, Kfast and Kslow were further used for statistical analyses. The goodness of fit was checked by the R^2 value, which was at least 0.990.

4. Statistical analysis

Data were analysed using the general linear model ANOVA procedure with the fixed effects of SN dose (n=3 levels) and SA dose (n=3 levels). The 2-way interaction term was only included in

the model when significant (P<0.05). The data of the batter and the pâté samples before and after display were analysed separately. Treatment means were compared using Tukey's post hoc test operating at a 5 % level of significance. All the statistical analyses were carried out by SAS Enterprise guide 4.

RESULTS

The average dry matter, fat and protein content was 46.6±0.2, 35.0±0.3 and 8.56±0.08 g/100g pâté respectively. The mean pH value of the batters was 6.35±0.04 and increased to 6.56±0.04 after heating (mean value of all pâtés before and after display).

Results concerning the batters are shown in Table 2.1. The initially added SN and SA doses affected significantly the nitrite and AA levels of the batters. Increasing SN and SA doses resulted in increasing nitrite and AA levels in the batters respectively. Also increasing DHAA levels were found with increasing SA doses. On the other hand, the 120 mg/kg SN dose resulted in a significantly lower AA level and higher DHAA level compared to the lower SN doses. Similarly, the nitrite concentration was significantly lower for the 1000 mg/kg SA dose compared to the 500 mg/kg SA dose. Overall, 53, 69 and 75 % of the initially added SA was measured as AA+DHAA in the batters containing 500, 750 and 1000 mg/kg SA respectively. A significant SN×SA interaction was found for the α-tocopherol levels, indicating that the effect of SA on α-tocopherol differed depending on the amount of SN initially added. However, no clear pattern was apparent. Also the effect of SA on lipid oxidation was affected by the SN dose: among the batters with 40 mg/kg SN, significantly lower TBARS values were found in samples with 750 mg/kg SA compared with batters containing 500 mg/kg SA, while for the batters with 80 mg/kg SN, a dose of 1000 mg/kg SA resulted in significantly higher TBARS values compared to 500 mg/kg SA. No effect of SA was found on lipid oxidation among the batters with 120 mg/kg SN. For the carbonyl content of the batters, higher SA doses resulted in higher carbonyl contents and no effect of the SN dose was seen.

Table 2.1 Effect of sodium ascorbate (SA) and sodium nitrite (SN) doses (mg/kg batter) on the content of nitrite, ascorbic acid (AA), dehydroascorbic acid (DHAA) and α-tocopherol, and on lipid oxidation (TBARS) and protein oxidation (carbonyls content), in raw batters

		Nitrite (mg/kg)	AA (mg/kg)	DHAA (mg/kg)	α-Tocopherol (mg/kg)	TBARS (mgMDA/kg)	Carbonyls (nmol DNPH/mg protein)
Main effects							
SN							
40[1]		11.8^c	393^a	130^b	7.91^b	0.90^c	3.94
80[1]		31.3^b	389^a	128^b	8.54^a	1.03^b	3.77
120[1]		66.8^a	304^b	199^a	8.53^a	1.35^a	4.02
	SA						
	500[2]	39.4^a	140^c	127^b	8.32^b	1.10	3.71^b
	750[2]	36.2^{ab}	370^b	152^{ab}	7.70^c	1.05	3.95^{ab}
	1000[2]	34.4^b	576^a	179^a	8.96^a	1.14	4.08^a
Interaction							
SN	**SA**						
40	**500**	13.7	148	116	7.87^{bc}	1.09^{bcd}	3.76
	750	11.3	417	111	7.96^{bc}	0.78^e	4.06
	1000	10.5	613	163	7.91^{bc}	0.85^{de}	4.01
80	**500**	35.9	170	112	9.24^a	0.93^{de}	3.42
	750	30.4	392	126	6.93^c	0.99^{cde}	3.82
	1000	27.7	605	146	9.45^a	1.18^{abc}	4.06
120	**500**	68.6	103	152	7.85^{bc}	1.28^{ab}	3.95
	750	67.0	301	218	8.21^b	1.38^a	3.96
	1000	64.9	509	227	9.54^a	1.38^a	4.16
RMSE[3]		2.60	25.7	23.0	0.25	0.06	0.22
P	**SN**	<0.001	<0.001	<0.001	0.003	<0.001	0.173
	SA	0.016	<0.001	0.006	<0.001	0.091	0.041
	SN×SA	-	-	-	<0.001	0.002	-

[a-e] Means within a column and within SN or SA (or within SN×SA in case the interaction term is significant) with no common superscript are significantly different at p<0.05;

[1] Means within SN dose across SA doses;

[2] Means within SA dose across SN doses;

[3] Root mean square error;

'-' refers to non significant (P>0.05) 2-way interaction, removed from the statistical model.

Results concerning the pâtés before display are shown in Table 2.2. Again clear dose-dependent responses were seen: lower SN doses resulted in lower nitrite levels in the pâtés and higher SA doses resulted in higher AA and higher DHAA levels. For the AA levels, a significant SA×SN interaction was observed and the dose-dependent response was clearly present within the three SN doses. Unlike the batters, nitrite was not affected by the SA dose. DHAA was affected by the SN dose with the DHAA level being significantly higher on the 120 mg/kg SN dose compared to the 80 mg/kg dose. Similar to the batters, significant SN×SA interactions were found for the α-tocopherol levels and TBARS values of the pâtés. No clear patterns were seen for the α-tocopherol levels. The TBARS values of the pâtés with 40 mg/kg SN, were significantly lower in samples with 750 and 1000 mg/kg SA compared with those containing 500 mg/kg SA, while no effect of SA was found for lipid oxidation among the pâtés with 80 and 120 mg/kg SN. No significant effects on the carbonyl content were found for the different SN and SA doses.

Table 2.2 Effect of sodium ascorbate (SA) and sodium nitrite (SN) doses (mg/kg batter) on the content of nitrite, ascorbic acid (AA), dehydroascorbic acid (DHAA) and α-tocopherol, and on lipid oxidation (TBARS) and protein oxidation (carbonyl content), in liver pâté before chilled display

		Nitrite (mg/kg)	AA (mg/kg)	DHAA (mg/kg)	α-Tocopherol (mg/kg)	TBARS (mgMDA/kg)	Carbonyls (nmol DNPH/mg protein)
Main effects							
SN							
40[1]		18.0[c]	341[b]	120[ab]	7.39	0.88[b]	4.03
80[1]		36.1[b]	382[a]	112[b]	7.73	0.80[b]	3.94
120[1]		67.5[a]	390[a]	128[a]	7.82	1.11[a]	3.76
	SA						
	500[2]	41.4	168[c]	95.0[c]	7.59[ab]	0.94	3.80
	750[2]	41.8	371[b]	125[b]	7.27[b]	0.92	3.94
	1000[2]	38.4	573[a]	141[a]	8.09[a]	0.93	3.99
Interaction							
SN	**SA**						
40	**500**	20.1	132[d]	92.3	6.88[bc]	1.03[ab]	3.93
	750	19.3	372[c]	129	7.90[ab]	0.78[c]	3.92
	1000	14.7	520[b]	139	7.40[abc]	0.85[bc]	4.23
80	**500**	40.5	192[d]	89.3	8.54[a]	0.77[c]	3.66
	750	36.0	368[c]	119	6.37[c]	0.76[c]	4.18
	1000	31.7	585[ab]	129	8.27[ab]	0.87[bc]	3.98
120	**500**	63.7	180[d]	103	7.34[abc]	1.03[ab]	3.81
	750	69.9	374[c]	127	7.55[abc]	1.21[a]	3.71
	1000	69.0	615[a]	154	8.58[a]	1.08[ab]	3.76
RMSE[3]		6.86	17.5	7.85	0.38	0.06	0.28
P	**SN**	<0.001	0.002	0.016	0.182	<0.001	0.290
	SA	0.661	<0.001	<0.001	0.016	0.839	0.517
	SN×SA	-	0.030	-	0.002	0.005	-

[a-d] Means within a column and within SN or SA (or within SN×SA in case the interaction term is significant) with no common superscript are significantly different at p<0.05;

'-' refers to non significant (P>0.05) 2-way interaction, removed from the statistical model;

[1] Means within SN dose across SA doses;

[2] Means within SA dose across SN doses;

[3] Root mean square error.

Results concerning the pâtés after chilled display are shown in Table 2.3. Again significant dose-dependent responses were found for SN and SA after chilled display on respectively the nitrite and AA levels. Similar to the results found in the batters, higher SA doses resulted in lower nitrite levels and lower SN doses resulted in higher AA levels. There was also a dose-dependent response of the DHAA levels to both the SN and SA doses, but the significant SN×SA interaction indicated that these responses varied within the different SN and SA doses. A significant SN×SA interaction was also found for lipid oxidation. Lipid oxidation was more intense at a SN dose of 40 mg/kg combined with 500 mg/kg SA compared to the other treatments, while a combination of 80 mg/kg SN and 500 mg/kg SA resulted in the lowest TBARS values. No significant effects were found for both the α-tocopherol levels and the carbonyls content of the pâtés after chilled display.

Table 2.3. Effect of sodium ascorbate (SA) and sodium nitrite (SN) doses (mg/kg batter) on the content of nitrite, ascorbic acid (AA), dehydroascorbic acid (DHAA) and α-tocopherol, and on lipid oxidation (TBARS) and protein oxidation (carbonyl content), in liver pâté after chilled display

		Nitrite (mg/kg)	AA (mg/kg)	DHAA (mg/kg)	α-Tocopherol (mg/kg)	TBARS (mgMDA/kg)	Carbonyls (nmol DNPH/mg protein)
Main effects							
SN	**SA**						
40[1]		13.1^c	217^{ab}	54.9^c	7.31	1.42^a	3.70
80[1]		29.3^b	229^a	66.3^b	7.43	1.20^b	3.61
120[1]		58.1^a	210^b	121^a	7.35	1.49^a	3.46
	500[2]	37.5^a	76.0^c	57.0^c	7.12	1.41^a	3.56
	750[2]	32.1^b	206^b	79.9^b	7.13	1.31^b	3.44
	1000[2]	30.8^b	374^a	105^a	7.85	1.39^{ab}	3.77
Interaction							
SN	**SA**						
40	**500**	15.0	77.1	40.7^f	7.18	1.68^a	3.54
	750	14.6	207	55.7^e	7.56	1.25^{cd}	3.47
	1000	9.76	367	68.4^d	7.20	1.31^{cd}	4.08
80	**500**	33.5	93.2	46.6^{ef}	7.85	1.08^d	3.60
	750	27.0	211	61.6^{de}	6.30	1.10^d	3.60
	1000	27.3	384	90.7^c	8.15	1.43^{bc}	3.63
120	**500**	64.2	57.8	83.7^c	6.34	1.48^{abc}	3.55
	750	54.9	201	122^b	7.53	1.57^{ab}	3.23
	1000	55.2	372	156^a	8.19	1.42^{bc}	3.60
RMSE[3]		2.36	9.06	2.72	0.94	0.06	0.27
P	**SN**	<0.001	0.009	<0.001	0.975	<0.001	0.333
	SA	0.001	<0.001	<0.001	0.339	0.036	0.133
	SN×SA	-	-	<0.001	-	<0.001	-

$^{a-f}$ Means within a column and within SN or SA (or within SN×SA in case the interaction term is significant) with no common superscript are significantly different at p<0.05;

[1] Means within SN dose across SA doses;

[2] Means within SA dose across SN doses;

[3] Root mean square error;

'-' refers to non significant (P>0.05) 2-way interaction, removed from the statistical model.

During 48 h chilled display, the a* value of all pâtés decreased in time, rapidly during the first 4 h followed by a slower decrease later onwards. The results obtainede from the fitted a* values to the bi-exponential model are shown in Table 2.4. The added amount of SA did not affect any model parameter. The rate constants Kfast and Kslow were affected by the SN dose. For Kfast significant higher values for samples containing 40 mg/kg SN were found, compared with 80 and 120 mg/kg SN, while for Kslow the samples with 40 mg/kg SN had only significant higher values compared to those containing 120 mg/kg SN. Although significant SN×SA interactions were found for Y0 and the plateau, it can be generalized that samples with 120 mg/kg SN showed lower Y0 values compared to the 40 or 80 mg/kg SN dose. A 80 mg/g SN dose resulted in higher plateau values compared to the 40 or 120 mg/kg SN dose. No significant differences between all treatments were found for %Fast.

Table 2.4 Effect of sodium ascorbate (SA) and sodium nitrite (SN) doses (mg/kg batter) in liver pâté during chilled display on model parameters derived from fitting a* values to a two-phase exponential decay curve

Colour		$Y0^4$	$\%Fast^5$	$Kfast^6$ ($\times 10^{-2}$)	$Kslow^6$ ($\times 10^{-2}$)	$Plateau^7$
Main effects						
SN	**SA**					
40^1		10.4^a	38.2	3.52^a	0.262^a	4.61^b
80^1		10.4^a	40.9	2.23^b	0.216^{ab}	4.89^a
120^1		10.1^b	39.7	2.19^b	0.171^b	4.37^c
	500^2	10.4	40.1	2.92	0.219	4.58
	750^2	10.3	40.3	2.56	0.221	4.67
	1000^2	10.3	38.4	2.47	0.209	4.62
Interaction						
SN	**SA**					
40	**500**	10.2^{bcd}	37.6	4.38	0.279	4.38^d
	750	10.5^{ab}	41.3	2.99	0.240	4.70^{bc}
	1000	10.5^{ab}	35.7	3.19	0.268	4.76^{bc}
80	**500**	10.6^a	41.1	2.23	0.210	4.88^{ab}
	750	10.5^{ab}	40.3	2.45	0.247	5.06^a
	1000	10.2^{bcd}	41.3	2.01	0.192	4.72^{bc}
120	**500**	10.3^{abc}	41.7	2.13	0.167	4.48^{cd}
	750	9.91^d	39.4	2.25	0.176	4.24^d
	1000	10.1^{cd}	38.1	2.21	0.168	4.39^d
RMSE³		0.19	12.5	0.00	0.098	0.16
P	**SN**	<0.001	0.813	0.031	0.022	<0.001
	SA	0.314	0.877	0.700	0.926	0.251
	SN×SA	<0.001	-	-	-	<0.001

[a-d] Means within a column and within SN or SA (or within SN×SA in case the interaction term is significant) with no common superscript are significantly different at p<0.05;

[1] Means within SN dose across SA doses;

[2] Means within SA dose across SN doses;

[3] Root mean square error;

[4] Initial a* value (intercept);

[5] Fraction of the span (from Y0 to plateau) accounted for by the faster of the two components.

[6] Rate constants, expressed in inverse minutes;

[7] Ultimate a* value after 48 h chilled display;

'-' refers to non significant (P>0.05) 2-way interaction, removed from the statistical model.
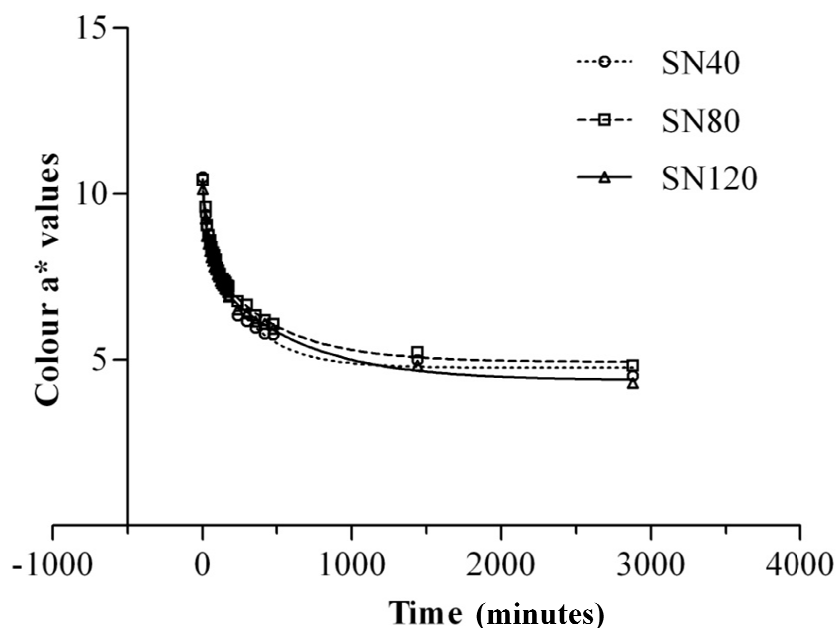
Figure 2.2. Two-phase exponential decay curve fitted to the mean colour a* values for 40, 80 and 120 mg/kg sodium nitrite (SN) across the sodium ascorbate doses.

The data for the batters and the cooked liver pâtés before and after display were not statistically compared, however, some trends were apparent. The manufacturing process of the liver pâtés, including the heating process, did not affect the AA and DHAA levels of the pâtés compared to the batters. However, after 48 h of chilled display considerable lower levels of these ingredients were found. On the contrary, the α-tocopherol content decreased during the manufacturing process, while no decrease during chilled display was found. Unexpectedly, the nitrite levels were lower in the batters compared with the pâté samples before chilled display and the TBARS values were higher in the batters compared with the pâtés. However, after chilled display the nitrite levels were lower and TBARS values were higher compared with the values of the pâtés before display. The carbonyl content did not change during the manufacturing of the pâtés, but decreased slightly during chilled display.

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DISCUSSION

Nitrite is a highly reactive chemical. When added to a meat system, it reacts with different components and a small amount of residual nitrite remains. In the present study, also during chilled display the residual nitrite levels continued decreasing. Sebranek et al. (1973) found that the residual nitrite decreased with time during display until a fairly constant low level was reached. In the batters, after manufacturing of the pâtés and after chilled display, the initially added amounts were reflected in the residual nitrite concentrations of the samples. However, the European Food Safety Authority (2003) concluded, based on several studies in which 0 to 300 mg/kg product SN was added, that there is no simple and direct relationship between the in-going and residual nitrite level. Fujimaki et al. (1975) found in a meat-curing model system that all nitrogen in nitrite, after curing and heating, is recovered as residual nitrite, nitrate, nitrosyl groups of denatured nitrosomyoglobin and gaseous nitrogen compounds. The distribution of these components however, depended on the concentrations of the remaining myoglobin, AA and nitrite in the model after heating. When AA is added to a cured meat product it accelerates all the reducing steps. Consequently, the formation of nitric oxide from nitrite is accelerated which can result in diminished residual nitrite levels in the product. Indeed, higher levels of SA in our study resulted in an increased nitrite depletion, similar to what Brown et al. (1974) found in cured hams. Unexpectedly, higher levels of residual nitrite were found after heating compared to the levels in the batters, while other studies have found a decrease of residual nitrite after heating (Gibson et al., 1984; Izumi et al., 1989; Okayama et al., 1991). Regrettably, as no other studies were found that report on increased nitrite concentrations after cooking, it was not possible to unravel a mechanism behind this observation. It is known that proteins in cured meats serve as a reservoir for NO and nitrosating agents that may be released during storage and cooking (Skibsted, 2011). One might therefore hypothesize that nitrite bound to proteins is similarly released during cooking.

In the batters, only 57 to 85 % of the added SA was recovered as AA and DHAA, taking into account that 11% of the weight of SA is sodium. In fact, independent of the initially added amount of AA, a similar absolute amount (about 152 ± 22 mg/kg) was not determined as AA or DHAA. Most probably, the electrophile AA and DHAA molecules reacted with ingredients like proteins and other nucleophile compounds, resulting in reaction products from which AA and DHAA as such could not be recovered. For the determination of AA and DHAA an extraction buffer consisting of 5 % methanol was used. However, according to Dodson et al. (1992) food products that contain very low levels of AA and DHAA, and are high in starch and/or fat should be extracted with 95 % ethanol in order to eliminate sample matrix interferences. Although AA and DHAA are not naturally present in the pâtés major ingredients, but were derived from SA added during manufacturing, some reaction products from AA and DHAA with other compounds, were probably not extracted due to the high fat content of the pâtés. Oxidation during chilled display may have further contributed to a decrease in AA. Importantly, the concentrations AA and DHAA were also affected by the initially added SN dose. This implies that SA not only influences the residual nitrite concentrations, but that also *vice versa*, SN influences the AA and DHAA levels. These reactions however, were not always dose-dependent. Although no α-tocopherol or α-tocopherol acetate was added during manufacturing, relatively high amounts of α-tocopherol were measured compared to other meat products (Bunnell et al., 1965), most likely due to the high α-tocopherol content of liver and pork fat (Gebert et al., 2006). After cooking, a significantly lower α-tocopherol content was observed in the pâtés. Also Bou et al. (2006) found significantly lower α-tocopherol concentrations in cooked chicken meat compared to raw meat. They suggested that cooking-related lipid oxidation could be the main factor responsible for the loss of α-tocopherol. Considering its antioxidant activity, α-tocopherol itself is subject to destruction by oxygen, giving rise to a number of products including quinones, dimers, trimers and epoxides (Bramley et al., 2000).

The dose of SA had an effect on the α-tocopherol content in the batters and pâtés before chilled display, but this effect depended on the amount of SN initially added. In the case of batters containing 120 mg/kg SN, higher levels of SA resulted in higher α-tocopherol concentrations, which was expected as AA reduces the semistable tocopheroxyl radical, resulting in the regeneration of α-tocopherol (Kitts, 1997). However, different results were found in samples with 40 or 80 mg/kg SN, which emphasizes the complex reactions and interactions taking place between nitrite, AA and α-tocopherol. These interactions are yet not fully elucidated.

Igene et al. (1985) showed that during heating nitrite decreases the release of non-heme iron and hypothesized that this effect decreases the catalysis of lipid oxidation. This could explain the lower TBARS values and lower AA levels of some pâtés before display compared to their batters. After chilled display, the lipid oxidation intensity depended both on the SN and SA dose. Morrissey & Tichivangana (1985) found higher TBARS values after 48 h of chilled display when lower nitrite concentrations (from 0 to 200 mg/kg SN) were added in cooked minced muscles of beef, pork and chicken. Dineen et al. (2001) found significantly higher TBARS values in low nitrite hams (25 mg/kg SN) compared to control hams (100 mg/kg SN) after 10 days of chilled display, while Sammet et al. (2006) did not find any effect of nitrite on TBARS during 8 weeks of chilled display in low-nitrite salami-type sausages containing 100, 50, 25 or 0 mg/kg SN and stored under protective atmosphere. Conversely, in the present study, higher TBARS values were found in the samples with 120 mg/kg SN compared to 80 mg/kg. The variety of meat product composition in these studies, all differently susceptible to lipid oxidation, could maybe explain these contrasting results. No antioxidant effect of SA was seen in pâtés containing 120 or 80 mg/kg SN after chilled display. Moreover, a pro-oxidant effect of SA was found in 80 mg/kg SN pâtés at the SA dose of 1000 mg/kg compared to the doses 750 or 500 mg/kg SA. Likewise, Sahoo & Anjaneyulu (1997) found higher TBARS values in ground buffalo meat when 600 mg/kg SA was added compared to 500 mg/kg. On the contrary, when

lowering the added SN dose to 40 mg/kg, an additional antioxidant effect of SA was found after chilled display in pâtés containing 750 and 1000 mg/kg SA compared with pâtés containing the conventionally used dose of 500 mg/kg SA.

Carbonyl compounds are formed as a result of the oxidative degradation of side chains from lysine, proline and arginine residues (Stadtman & Levine, 2003). In this study, the carbonyl values of the pâtés were similar to those reported by Estévez et al. (2005b), Estévez et al. (2006b) and Armenteros et al. (2009) for liver pâté. The carbonyl content was not affected during heating, while it was expected that due to the increased temperature the formation of reactive oxygen or nitrogen species was enhanced and oxidation would occur. Ganhão et al. (2010b) found increased carbonyl compounds in cooked porcine burger patties after heating raw patties for 18 minutes in an oven of 170 °C. Maybe, the applied temperature in the present study, in combination with a relatively short exposure time, was too low to induce protein oxidation in liver pâté. Gatellier et al. (2010) observed an increase in carbonyl content in fresh meat after heating up to 207 °C, but no increase was found after heating at 65 °C or 96 °C. An increase of protein carbonyls was also expected during chilled display, analogous to what was observed in other studies (Lund et al., 2007; Ganhão et al., 2010b; Ganhão et al., 2010c), but on the contrary, a small decrease in carbonyl groups was found. Possible reasons could be the shorter chilled display period and the difference in meat products, as the above mentioned studies investigated cooked porcine burger patties and raw beef patties during 12 and 6 days chilled display respectively. As reviewed by Estévez (2011), carbonyl compounds are reactive and may disappear as a result of their participation in other reactions. A decrease in carbonyl content was seen in muscles homogenates after *in vitro* induced oxidation, using different metal-catalyzed oxidation systems (Martinaud et al., 1997; Batifoulier et al., 2002; Mercier et al., 2004). Induced oxidation is more intense compared to 48 h of chilled display conditions, but because of the high

concentrations of iron in liver pâté, it could be hypothesized that more intense oxidation and the same kind of reactions occurred in the present study.

From our results, SN did not affect the carbonyl content of the pâtés and as far as we know, this study is the first to investigate the effect of SN on protein oxidation in meat products. As nitrite chelates iron (Skibsted, 2011) and transition metals are essential to the formation of major protein carbonyls from lysine, arginine and proline (Estévez, 2011), it could be hypothesized that SN has an effect on carbonyl formation during protein oxidation. On the other hand, SN also acts as an antioxidant by sequestering oxygen (Honikel, 2008), but the formation of protein carbonyls does not require molecular oxygen (Estévez, 2011). Regrettably, no other protein oxidation markers were analysed in the present study to investigate this further. An interesting marker would be 3-nitrotyrosine, a protein oxidation product originating from tyrosine residues (Means & Feeney, 1998). According to Woolford et al. (1976), the major nonheme muscle protein, myosin, has the ability to bind appreciable amounts of nitrite with resulting modifications of the protein, mainly in 3-nitrotyrosine. For SA, higher carbonyl contents were found in batters containing 1000 mg/kg SA compared with batters containing 500 mg/kg. It is well known that AA can act as a pro-oxidant by reducing Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+}, thereby increasing the pro-oxidant activity of these metals (Morrissey et al., 1998). Lund et al. (2007) also found a pro-oxidant effect of ascorbate:citrate (1:1) during chilled storage of minced beef patties in combination with modified atmosphere packaging. They assigned this observation to the fact that protein carbonyl compounds are primarily formed through metal catalysed oxidation, and that the presence of ascorbate enhances the conversion of some amino acids to carbonyl derivatives due to ascorbate-driven redox cycling of metal ions such as Fe^{2+} and Cu^{+} (Amici et al., 1989).

The results revealed that SA and SN had overall an effect against lipid oxidation, but not against protein oxidation. According to Lund et al. (2007), the efficiency of an antioxidant is dependent on the rate of reaction between the antioxidant and the radical intermediates in the autocatalytic

process relative to other possible reactions of the radical intermediates with other oxidation substrates present in the product. Hence, the substrate radicals formed during oxidation have to be relatively long-lived in order to be quenched by antioxidants. Although the exact mechanism of antioxidants towards protein oxidation is not yet elucidated (Lund et al., 2011), one might hypothesize that the conditions prevailing in the pâtés of the present study gave rise to the formation of protein radicals that were more reactive than lipid radicals, allowing SA and SN to exert antioxidant protection only against lipid oxidation but not against protein carbonyl formation. However, this remains to be further investigated.

Since meat purchasing decisions are influenced by colour more than any other quality factor (Mancini & Hunt, 2005), good colour formation is important. In addition, as liver pâté is highly susceptible to oxidation, the colour shelf life of liver pâté is very short. The 48 h of display used in the present study simulates the use of liver pâté by consumers: after opening the package, the liver pâté is exposed to light and air and is generally consumed within a few days. From previous results (unpublished data), it was found that an immediate drop in redness of the pâtés takes place during the first hours of chilled display. Therefore, in the present study, the a* values were measured every 10 minutes for the first 3 h. This loss of redness displayed a non-linear two-phase exponential decay pattern (Figure 2.1). However, little is known about the biochemical mechanisms related to this two-phase colour fading. Other studies investigating the colour of pork liver pâté did not look at the a* values in detail during the first hours of chilled display (Perlo et al., 1995; Estévez et al., 2005b; Estévez et al., 2006b; Kaack et al., 2006). In the present study no differences in the initial a* values were found between the different SA treatments, pointing out that increasing the conventional 500 mg/kg SA dose to 750 or 1000 mg/kg gives no additional colour gain. Importantly, the initial a* values of samples containing 120 mg/kg SN were lower than those with 40 and 80 mg/kg, while the opposite was expected. Apparently, using 40 mg/kg SN is sufficient to have proper colour formation. However, this dose was less effective

in retaining the redness of the samples during chilled display compared to the 80 and 120 mg/kg SN doses. No benefit of increasing the dose of SA to using 750 or 1000 mg/kg on the stability of the cooked cured colour was found either.

CONCLUSIONS

Lowering the use of nitrite in liver pâté to 80 or even 40 mg/kg SN should be possible without facing major problems concerning the oxidative stability of the liver pâtés. Additional research should be done to verify if these lower doses still have a sufficient protective effect against microbiological risks. Also, sensory research on the acceptability of low-nitrite liver pâtés is warranted. Due to the multifunctional roles of nitrite, an approach where several additives are used to replace nitrite will be necessary. Sodium ascorbate is one of these potential additives.

ACKNOWLEDGEMENTS

The ingoing sodium nitrite level can be lowered to 40 mg/kg in liver pâté, without major problems concerning the oxidative stability (CH2).

What happens when the ingoing nitrite content is lowered to 20 mg/kg using nitrite from a pre-converted plant extract?

<table>
<tr><td>Part I Lowering the ingoing nitrite dose</td><td>Part II Increasing the n-3 PUFA content</td></tr>
<tr><td>CH1 With a dog rose extract</td><td>CH5 Role of n-3 PUFA source</td></tr>
<tr><td>CH2 With sodium ascorbate</td><td>CH6 Effect on sensory quality</td></tr>
<tr><td>CH3 With a pre-converted extract</td><td>CH7 Role of α-tocopherol in feed</td></tr>
<tr><td>CH4 Nitrite and protein oxidation?</td><td>CH8 Effect on health</td></tr>
<tr><td colspan="2" align="center">CH9 General discussion and future prospects</td></tr>
</table>

SHELF LIFE STABILITY OF REDUCED NITRITE LIVER PÂTÉS USING

A PRE-CONVERTED PLANT EXTRACT AS SOURCE OF NITRITE

ABSTRACT

The effect of reduced nitrite liver pâtés using a pre-converted plant extract as source of nitrite was investigated on colour stability (a* values), protein oxidation (carbonyls) and lipid oxidation (TBARS and hexanal). Five treatments were prepared: 20, 45 and 90 mg/kg nitrite from a pre-converted extract, a negative control without nitrite and a positive control with 120 mg/kg sodium nitrite. Samples were subjected to illuminated chilled display for nine days. Decreasing the ingoing nitrite dose resulted in lower residual nitrite levels, but did not affect the colour formation and colour stability. Inconsistent results for the treatment effects on the protein carbonyls content of the liver pâtés were found. Compared to the positive control, lipid oxidation was lower in the treatments with 45 and 90 mg/kg ingoing nitrite at the end of display, which illustrates that the antioxidant effect of sodium nitrite was partly replaced by other compounds present in the extract.

INTRODUCTION

Worldwide, nitrite is used in meat products for its multifunctional properties. It is a highly reactive molecule responsible for cured meat colour, cured flavour, lipid oxidative stability and bacterial inhibition (Honikel, 2008). However, this reactivity is also a concern, as it is related to the potential formation of carcinogenic nitrosamines in cured meat or during digestion (Sindelar & Milkowski, 2011). Although it is not clear to what extent the nitrite used in curing salt is related to this issue, public concern on conventionally produced foods raises, and there is a shift in consumers preferences towards the consumption of natural foods (Sebranek & Bacus, 2007). As a result, meat scientists and meat producers are challenged to search for reliable alternatives for conventional cured meat products.

In this context, meat products are processed without the use of synthetic sodium nitrite, but to which nitrite is added through natural sources. This nitrite is mostly formed from the conversion of nitrate, present in vegetables and fruits, by nitrate-reducing micro-organisms during manufacturing. Cherry powder in emulsified cooked sausages (Terns et al., 2011), celery with carrot concentrate in cured cooked sausage (Magrinya et al., 2012), celery juice in ham (Sindelar et al., 2007a) and celery juice in emulsified cooked sausages (Sindelar et al., 2007b) have been tested as indirect curing agents. With the addition of a nitrate reducing bacterial starter culture, all of them have been found effective alternatives to traditional curing processes. The drawback of this approach is the lack of knowledge about the doses of nitrite actually added during meat processing and because of the high reactivity of nitrite, it is impossible to deduce the ingoing amount from the residual nitrite after production. For this reason, pre-converted powders and extracts have become commercially available for the production of naturally cured meat products (Krause et al., 2011). These pre-converted powders and extracts are produced by combining nitrate-reducing organisms with vegetable products with a sufficient amount of nitrate, resulting in a product with a known amount of nitrite. By this way, a more controlled addition of nitrite

from a natural source is possible. However, these meat products still contain residual nitrite, so lowering the ingoing nitrite levels remains of interest.

Studies investigating natural curing are generally conducted in cooked sausages and ham, while liver pâté is hardly investigated. Liver pâté is widely consumed in many countries and the increasing demand of natural meat products therefore also concerns liver pâté. In addition, as the main ingredient is liver instead of muscle tissue, one might expect a different effect of natural curing processes on the oxidative stability of this product compared to other cooked and cured meat products. Liver pâté is very prone to oxidation due to its chemical composition, e.g. high in fat and iron content, and manufacturing process such as mincing and cooking (Estévez et al., 2007a). These oxidation processes can lead to colour deterioration (Mancini & Hunt, 2005), lipid oxidation with the occurrence of lipid oxidation products such as malondialdehyde and hexanal (Morrissey et al., 1998) and protein oxidation, with the formation of e.g. protein carbonyl compounds (Lund et al., 2011). Liver pâté is therefore an interesting product when studying the effect of natural curing on oxidation processes.

The objective of this study is to investigate the colour stability, lipid oxidation and protein oxidation processes in reduced nitrite liver pâtés using a pre-converted vegetable extract as source of nitrite.

MATERIALS AND METHODS

1. Manufacturing of the liver pâtés, experimental design and sampling procedure

A pre-converted extract (PE, Herba Pure) was supplied by RAPS (Beringen, Belgium) and consists of a liquid kombucha tea extract containing flavouring spices. The nitrite content of PE was analysed and found to be 1.25 g/kg. Three doses of the extract were added to the

experimental pâtés: 17.5, 35 and 70 g PE/kg pâté, corresponding to approximately 20, 45 and 90 mg nitrite/kg pâté (PE20, PE45 and PE90 treatments respectively). Additionally, a negative control (NEG0) without added $NaNO_2$ or PE, and a positive control (POS120) consisting of 120 mg $NaNO_2$ mg/kg liver pâté was included in the trial. For each treatement 2 kg of batter was made. The composition of the pâté batters was (g/kg): 300 g liver, 400 g of back-fat, 300 g water (in the case of the addition of PE, the amount of water added was 300 g minus the amount of PE added), 18 g sodium chloride, 0.5 g sodium ascorbate, 10 g sodium caseinate, 5 g dextrose. The ingredients were purchased from Kerry Ingredients and Flavours (Bornem, Belgium).

The pâtés were manufactured as described by Vossen et al. (2012a) with minimal modifications. Two different batches were prepared on two subsequent days. Each day all treatments were prepared from the same homogenized common ingredients. Before manufacturing the pâtés, separate mixtures of liver and subcutaneous fat from several commercial slaughter pigs were made. Each mixture was divided in five parts to be used for the different treatments. First, the liver mixture was minced for 8 minutes at 3000 rpm (Stephan vertical cutter-mixer, model UM12-F/3). Sodium chloride and, in the case of POS120, 120 mg/kg $NaNO_2$ were added and the cutting process was continued under vacuum for 2 minutes at 1200 rpm. The fat mixture was scalded for 20 minutes in boiling water. The fat was then minced and homogenized with sodium caseinate and broth (i.e. liquid where the fat had previously been boiled in) for 5 minutes at 51 °C in the Stephan vertical cutter mixer. Thereafter, the liver with the curing salts was added and the whole mixture was homogenized for 3 minutes until the raw batter reached 40 °C. The finished batters were manually distributed into metal cans of 250 g of capacity, until completely full. Cans were hermetically closed with a can sealing machine (Indosa, type M160). The cans were cooked in saturated steam conditions at 75 °C for 90 minutes. Subsequently, the cans were rapidly cooled in an ice bath and stored in the dark at 4 °C. After 7 weeks, six cans per treatment were opened and two slices of 2 cm thickness were sampled from each can after removing the

top and the bottom layer. Three slices per treatment and per batch were immediately vacuum packed and stored at -80 °C (Day 0). Six other slices per treatment and per batch were wrapped in an oxygen permeable polyethylene film and placed in an illuminated chilled cabinet (1000 lux, 4 °C). After 5 and 9 days of display, the polyethylene film was removed and the samples were vacuum packed and stored at -80 °C until further analysis.

2. Composition analysis

Dry matter, crude protein and crude fat content were analyzed in duplicate on unexposed samples according to the ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods, respectively for each treatment and each batch (n=4). The results are expressed as g/100g of pâté. Nitrite content was determined on the Herba Pure extract and unexposed liver pâté samples according to Zuo et al. (2006) with some modifications as described by Doolaege et al. (2012) using a HPLC (Agilent 1200 series) with DAD detector. The column employed was ZORBAX Eclipse XDB-C18; 4.6×150 mm, 5 μm (Agilent) and the elution was performed at a flow rate of 0.4 ml/min following a gradient. Detection was done by UV absorption measurement at 225 nm. The peak area of NO_2^- (retention time 25 min) was read and the quantification was done by comparison with the peak areas obtained from a standard solution of sodium nitrite. Analyses were carried out in duplicate and results were expressed as mg $NaNO_2$/kg pâté.

3. Colour stability

Colour coordinates (CIE L*a*b*colour system 1976) were measured with a HunterLab Miniscan Minolta XE plus spectrocolorimeter (light source of D65, standard observer of 10°, 45°/0° geometry, 1 inch. light surface, white standard). Three slices per treatment and per batch were measured (n=6 per treatment), every 10 minutes for the first 3 h, every hour from 3 h until 7 h

and after 1, 2, 5 and 9 days of illuminated display in the chilled cabinet. The colour fading from day 0 until day 2, measured as a decline in a* values, was fitted to a non-linear, two-phase exponential decay curve (GraphPad Prism6, Demo 2014) using the equation:

$$Y = plateau + A \times exp(-Kfast \times X) + B \times exp(-Kslow \times X)$$

with $A = (Y0 - plateau) \times \%Fast \times 0.01$

                    $B = (Y0 - plateau) \times (100 - \%Fast) \times 0.01$

                    $Y0$ = initial a* value (intercept)

                    Plateau = the ultimate a* value after 48 h of display

                    Kfast and Kslow (expressed in inverse minutes) = the two rate constants

                    %Fast = the fraction of the span (from Y0 to plateau) accounted for by the faster of the two components.

The measurements were fitted separately per replicate, and the fitted parameters for Y0, plateau, %Fast, Kfast and Kslow were further used for statistical analyses. The goodness of fit was checked by the R^2 value, which was at least 0.99. NEG0 was not fitted to a two-phase exponential decay curve as a linear decrease was seen for these samples.

4. Lipid oxidation

Lipid oxidation was assessed by the measurement of malondialdehyde and hexanal. Malondialdehyde, a secondary oxidation product, was determined following a TBARS method using extraction with perchloric acid (Ventanas et al., 2006). Malondialdehyde forms a coloured complex with 2-thiobarbituric acid (TBA) which was determined spectrophotometrically at 532 nm. The analysis was carried out in duplicate for each treatment and each batch (n=4) and results are expressed as µg malonaldehyde/g pâté sample.

Hexanal was assessed by SPME-GC/MS (Solid Phase Micro-Extraction – Gas Chromatography/Mass Spectroscopy), based on Ventanas et al. (2006) and Fernando et al. (2003) with some modifications. Hexanal was extracted from the headspace using a carboxen-polydimethylsiloxane (CAR/PDMS) fiber (85 µm thickness) (Supelco, Bellefonte, Pennsylvania, USA). One g of homogenised pâté with 3 ml deionized water was put in a 10 ml vial and after gently swirling hexanal was extracted in a heating block for 60 min at 37°C. Hexanal was analyzed using a gas chromatograph (Agilent model 6890N) coupled to a mass-selective detector (Agilent model 5973, Agilent Technologies, Diegem, Belgium). Compounds were resolved on a HP-5 column (30 m × 250 µm × 1 µm, 5% phenyl methyl siloxane, Agilent Technologies, Diegem, Belgium), at an inlet temperature of 280°C. Hydrogen flow was 1.1 ml/min and the temperature program was as follows: 40°C for 10 min, increase at 5°C/min to 190°C, increase at 30°C/min to 250°C and hold for 5 min. N-alkanes were run under the same conditions to calculate the Kovats index (KI). Hexanal was identified by comparing its mass spectra with those contained in the NIST05 mass spectral library and by comparison of KI with those reported in literature. Area of peaks was measured by integration of the total ion current of the spectra or by calculation of the total area based on integration of a single ion. Samples were analyzed in quadruplicate per treatment and per batch (n=8) and results are provided in arbitrary area units (AAU$\times 10^6$).

5. Protein oxidation

Total protein carbonyl content was quantified using dinitrophenylhydrazine (DNPH) according to Ganhão et al. (2010b). A covalent reaction between protein carbonyls and DNPH leads to the formation of 2,4-dinitrophenyl hydrazones. Total carbonyl content was quantified spectrophotometrically at 370 nm, using a molar absorption coefficient of 21.0 (mM·cm)$^{-1}$ and expressed as nmol DNPH incorporated/mg protein. The protein concentration used to calculate

the carbonyl content was the one obtained from the crude protein analysis. The analysis was carried out in duplicate for each treatment and each batch (n=4).

6. Statistical analysis

Data obtained from the colour measurements were analysed using one-way ANOVA considering 'treatment' as fixed effect. Lipid and protein oxidation measurements were analysed using the general linear model ANOVA procedure with the fixed effects of treatment, storage day and the interaction term. The interaction term was excluded from the model when not significant (P>0.05). When the interaction term was found significant, a new factor called "experimental unit" was computed combining treatment and storage day and one-way ANOVA considering "experimental unit" as fixed effect was conducted. Significant differences were tested using Tukey's post hoc test and significance was determined at p<0.05. All the statistical analyses were carried out by SPSS Statistics 22.0.

RESULTS

1. Composition analysis

The crude composition did not differ among treatments (P>0.05). The mean values ± standard deviations across treatments were 39.83±0.41, 25.27±0.35 and 11.85±0.11 g/100 g pâté for dry matter, fat and protein content respectively.

The residual nitrite concentration was 0.45±0.64, 5.63±3.8, 16.1±0.4, 47.8±9.6 and 53.3±3.38 mg/kg pâté for respectively NEG0, PE20, PE45, PE90 and POS120. Higher residual nitrite concentrations were found in POS120 and PE90 samples compared to the other treatments (P<0.05).

2. Oxidative stability

Colour stability results are shown in Table 3.1. At the start of the trial (day 0), the highest a* values were found for the POS120 and PE45 treatments and intermediate a* values for the PE20 and PE90 treatments, while the a* values of NEG0 samples were the lowest and significantly different from all other treatments ($P<0.05$). During illuminated chill display, the a* value of all pâté's decreased in time. The a* values decreased rapidly in the POS120 and the experimental pâté's, while a slow decrease was seen for NEG0, resulting in higher a* values for NEG0 at day 2 and 9 ($P<0.05$) compared to the other samples.

In all samples, except for NEG0 samples, this fading was fast during the first 4 hours and slowed down further onwards. Therefore, data until day 2 were fitted to a two-phase exponential model. The a* values at day 0 and the Y0 values, and the a* values at day 2 and the plateau values were comparable respectively, indicating that the model fitted well to the data. Kfast and Kslow reflect the rate of colour change during display. Kfast varied between 1.37 and 2.69×10^{-2}/min, meaning that the a* value decreased between 0.8 and 1.6 units per hour. No significant differences between POS120 and the experimental pâtés were found for Kfast ($P>0.05$), while for Kslow a significantly lower ($P<0.05$) value was found for PE90 samples, meaning that at the end of two days of display the a* value of PE90 samples decreased slower compared to the others. No significant differences between treatments were found for %Fast ($P>0.05$).

At the start of the trial, L* and b* values did not differ ($P>0.05$) between the PE treated and POS120 samples (data not shown), indicating no colour effect, except for the redness, of the pre-converted extract on the experimental pâtés.

Table 3.1. Effect of nitrite originating from a pre-converted extract in liver pâté during illuminated chill display on measured a* values and results obtained from fitting a* values to a two-phase exponential decay curve

	NEG0	PE20	PE45	PE90	POS120	$p_{treatment}$
a* at day 0	8.80±0.67[d]	10.3±0.2[c]	12.1±0.6[a]	10.9±0.5[bc]	11.6±0.3[ab]	<0.001
a* at day 2	7.36±0.61[a]	5.38±0.19[c]	6.23±0.68[b]	6.30±0.09[b]	6.35±0.27[b]	<0.001
a* at day 9	4.30±0.26[ab]	2.88±0.13[c]	3.88±0.41[b]	4.63±0.16[a]	4.07±0.26[b]	<0.001
Y0[1]	-	10.2±0.2[b]	12.0±0.6[a]	10.7±0.1[b]	11.5±0.4[a]	<0.001
%Fast[2]	-	44.2±20.9	39.6±8.8	62.5±7.5	37.6±16.3	0.089
Kfast (×10^{-2})[3]	-	2.22±1.33	2.69±1.35	1.37±0.20	2.17±0.79	0.343
Kslow (×10^{-2})[3]	-	0.346±0.252[a]	0.338±0.063[a]	0.062±0.046[b]	0.366±0.129[a]	0.028
Plateau[4]	-	5.29±0.37[b]	6.41±0.55[a]	5.66±0.64[ab]	6.41±0.24[a]	<0.001

NEG0: negative control without nitrite; PE20, PE45 and PE90: experimental pâtés with respectively 20, 45 or 90 mg/kg ingoing nitite originating from a pre-converted extract. POS120: positive control with 120 mg/kg ingoing sodium nitrite;

[a-d] Different letters indicate significant differences between treatment means at p<0.05;

[1]Initial a* value (intercept);

[2]Fraction of the span (from Y0 to plateau) accounted for by the faster of the two components;

[3]Rate constants, expressed in inverse minutes;

[4]Ultimate a* value after 48 h illuminated chill display.

The lipid stability of the liver pâte's during illuminated chill display was measured by TBARS and hexanal content (Table 3.2). During the nine days of illuminated chill display, lipid oxidation progressed as TBARS values of all treatments, except for PE90, increased significantly ($P<0.05$). Within day 5 and day 9, higher TBARS values ($P<0.05$) were found in NEG0 and PE20 samples, compared to the other treatments.

Higher hexanal values were measured at day 9 compared to day 0 ($P<0.05$), except for liver pâtés from the NEG0 treatment which had considerably high hexanal values at all time points. The treatment $\times$ display time interaction was significant, meaning that the increase in lipid oxidation products with time depended on the treatment. After 9 days of illuminated chill display, two and three fold lower hexanal values were found in PE45 and PE90 samples respectively, compared to POS120 ($P<0.05$). A dose effect of the PE against lipid oxidation was seen, as higher PE doses resulted in lower TBARS and hexanal values in the experimental pâtés.

Protein oxidation was measured by protein carbonyl compounds and is summarized in Table 3.2. During the illuminated chill display, across treatments, higher carbonyl compounds were observed at day 5 and day 9 compared to day 0 ($P<0.05$). At the three time points, lower carbonyl compounds were found in the POS120 samples compared to all other treatments, but this difference was only significant between the PE90 and POS120 treatments across days of display ($P<0.05$). There was no interaction between treatment and days of chilled display ($P>0.05$).

Table 3.2. TBARS values (mg/kg pâté), hexanal formation (AAU $\times 10^6$) and protein carbonyl content (nmol carbonyl compounds/mg protein) in liver pâté with added nitrite from a pre-converted extract during illuminated chill display

	Day	NEG0	PE20	PE45	PE90	POS120	*Mean*
TBARS[1]	**0**	0.435 ± 0.06^c	0.416 ± 0.034^c	0.337 ± 0.054^b	0.263 ± 0.076	0.262 ± 0.029^b	*0.34*
	5	$2.67\pm0.64^{b,x}$	$2.19\pm0.34^{b,x}$	$1.03\pm0.09^{a,y}$	0.675 ± 0.116^{yz}	$0.606\pm0.150^{b,z}$	*1.35*
	9	$4.07\pm0.19^{a,w}$	$3.11\pm0.35^{a,x}$	$1.53\pm0.14^{a,y}$	0.840 ± 0.055^{z}	$1.16\pm0.15^{a,yz}$	*2.14*
	Mean	*2.39*	*1.85*	*0.97*	*0.59*	*0.68*	
Hexanal[2]	**0**	164 ± 55^x	$6.55\pm2.37^{c,y}$	$5.86\pm2.17^{b,y}$	$5.00\pm0.00^{b,y}$	$5.00\pm0.00^{b,y}$	*37.3*
	5	163 ± 48^w	$78.4\pm18.9^{b,x}$	$48.3\pm14.1^{a,xy}$	$20.1\pm4.4^{a,y}$	$6.28\pm1.59^{b,z}$	*61.5*
	9	152 ± 15^x	$155\pm17^{a,x}$	$57.1\pm18.6^{a,z}$	$32.3\pm7.5^{a,z}$	$102\pm19^{a,y}$	*100*
	Mean	*160*	*80.3*	*37.1*	*19.1*	*37.6*	
Carbonyls[3]	**0**	1.76 ± 0.51	1.68 ± 0.47	1.87 ± 0.24	1.94 ± 0.64	1.47 ± 0.61	*1.74[b]*
	5	2.81 ± 0.14	2.83 ± 0.31	2.60 ± 0.41	2.85 ± 0.42	2.26 ± 0.53	*2.65[a]*
	9	2.93 ± 0.36	2.76 ± 0.21	2.90 ± 0.28	2.90 ± 0.20	2.44 ± 0.28	*2.79[a]*
	Mean	*2.50[ab]*	*2.34[ab]*	*2.46[ab]*	*2.56[a]*	*2.06[b]*	

NEG0: negative control without nitrite; PE20, PE45 and PE90: experimental pâtés with respectively 20, 45 or 90 mg/kg ingoing nitite originating from a pre-converted extract. POS120: positive control with 120 mg/kg ingoing sodium nitrite;

[a-c] Effect of display: values with a different letter within a column are different (P<0.05);

[w-z] Effect of treatment: values with a different letter within a row are different (P<0.05);

[1]$P_{treatment}$<0.001; $P_{display}$ <0.001; $P_{treatment \times display}$<0.001;

[2]$P_{treatment}$<0.001; $P_{display}$ <0.001; $P_{treatment \times display}$<0.001;

[3]$P_{treatment}$=0.019; $P_{display}$ <0.001; $P_{treatment \times display}$: not significant.

DISCUSSION

Using nitrite from a pre-converted extract and lowering the ingoing nitrite amount to even 20 mg/kg pâté did not affect colour formation of the liver pâtés. Vossen et al. (2012a) and Doolaege et al. (2012) also found proper colour formation in liver pâté with 80 and 40 mg/kg sodium nitrite. However, these studies found inferior colour stability of pâtés with 40 mg/kg sodium nitrite, which is not the case in the present study. Generally, an amount of 40 mg/kg ingoing nitrite is considered to be sufficient for colour-fixing purposes and to achieve the expected cured meat appearance (USDA, 1995), but the present results show that for liver pâté even lower concentrations are applicable. The pâtés with lower amounts of nitrite from the pre-converted extract showed similar colour stability as the control pâtés with even higher concentrations of ingoing sodium nitrite. It is hypothesized that additional colour stability was achieved from the added spices in the pre-converted extract. One should remember that 33% of the weight of sodium nitrite belongs to sodium, which implies that the actual amount of added nitrite in the positive control samples would be 80 mg/kg NO_2^-. This could explain the similar residual nitrite levels in the positive control samples and the PE90 experimental samples. However, PE20 and PE45 pâtés had similar colour stability and significantly lower residual nitrite concentrations compared to the sodium nitrite treated samples.

The initial values found in the present study for TBARS, hexanal and carbonyl compounds compare well with those found by Doolaege et al. (2012), Vossen et al. (2012a) and Pateiro et al. (2014) in liver pâté. From the results a clear antioxidant effect of the pre-converted extract against lipid oxidation was found. Moreover, better antioxidant properties in pâtés with 90 mg/kg nitrite from pre-converted extract and a similar residual nitrite content were found compared to pâtés treated with 120 mg/kg sodium nitrite. As the added amount of sodium ascorbate was equal for all treatments, this finding implies that the spices present in the pre-converted extract also have antioxidant properties against lipid oxidation. Regrettably, little is known about the spices

in the commercial pre-converted extract based on kombucha. Kombucha is composed of two portions: a floating cellulose pellicle layer and the sour liquid broth. It is also frequently called "tea fungus", which is the most usual name for a symbiotic growth of bacteria and various yeast strains cultured in sugared tea. Bacteria and fungus present in kombucha form a powerful symbiosis able to inhibit the growth of potential contaminating bacteria (Mo et al., 2008). Although scarce scientific information is available concerning the composition and the effects of kombucha, it is considered a healthy drink and therapeutic agent in diseases (Dufresne & Farnworth, 2000) and also free-radical scavenging activity has been reported (Jayabalan et al., 2008). It could be speculated that the extract contains phenolic compounds with antioxidant properties against lipid oxidation, but this was not confirmed by the supplier.

On the other hand, on average higher carbonyl compounds were found in pâtés containing the pre-converted extract at the highest dose compared to the control with 120 mg/kg sodium nitrite, which suggests that those compounds present in the pre-converted extract do not exhibit an antioxidant effect against protein oxidation. It was hypothesized that the extract contains phenolic compounds. The antioxidant activity of phenolic compounds is attributed to their free radical scavenging and metal chelating activities, in which hydroxyl groups attached to phenolic rings play an important role. However, during oxidation of these antioxidants, hydroxyl forms can convert to their corresponding pro-oxidant quinone structures. The quinone derivatives of phenolic compounds, formed in the presence of transition metals, are able to react with the δ-amino group of alkaline amino acids (lysine, arginine and/or proline), catalyze the oxidative deamination of the original amino acid and yield, eventually, in the corresponding carbonyl compound. The overall pro- or antioxidant effect displayed by plant phenolics against protein oxidation might therefore be the result of the balance between both forms (Estévez & Heinonen, 2010). Other studies also reported an effective antioxidant activity of extracts against lipid

oxidation, while no or less activity against protein oxidation was found (Estévez, 2006a; Haak et al., 2009; Rodriguez-Carpena et al., 2011; Vossen et al., 2012b; Cando et al., 2014).

The increase in carbonyl content between day 0 and day 9 of display was comparable for all treatments, suggesting that the residual nitrite content after processing had no protective effect against protein oxidation. On the other hand, the carbonyl content was at all time points numerically lower in the POS120 samples compared to all other treatments, suggesting some protective effect of nitrite curing against protein oxidation in liver pâté. From chapter 2 it was found that lowering the ingoing nitrite dose has no effect on protein carbonyls content in liver pâté during chilled display, which is in accordance with Villaverde et al. (2014). These authors reported that nitrite has a negligible effect on protein carbonyl formation in myofibrillar protein isolates. On the other hand, Van Hecke et al. (2014) found less carbonyl formation during *in vitro* digestion of nitrite-cured meat products compared to uncured meat products. Regarding the liver pâté's treated with the pre-converted extract, the combination of nitrite with other compounds from the extract resulted in similar protein oxidation as the negative control. It seems that the assumed antioxidant effect of nitrite was compensated by the pro-oxidant properties of the extract compounds. To our knowledge, except for Villaverde et al. (2014), no other in-depth studies have been performed investigating the effect of nitrite against protein oxidation in meat proteins and more research in this respect is needed.

CONCLUSIONS

This study showed that the ingoing nitrite content for liver pâté can be lowered without major changes in colour parameters in terms of colour formation and colour stability. Less lipid oxidation occurred in the pâtés prepared with pre-converted extracts, which seemed to result from other compounds present in the extract. The role of nitrite in the formation of protein carbonyls was unclear and needs more research.

ACKNOWLEDGMENTS

No consensus on the role of nitrite was found for protein oxidative stability during storage (CH3)

Is nitrite a pro- or antioxidant against protein oxidation?

Part I Lowering the ingoing nitrite dose	Part II Increasing the n-3 PUFA content
CH1 With a dog rose extract	CH5 Role of n-3 PUFA source
CH2 With sodium ascorbate	CH6 Effect on sensory quality
CH3 With a pre-converted extract	CH7 Role of α-tocopherol in feed
CH4 Nitrite and protein oxidation?	CH8 Effect on health
CH9 General discussion and future prospects	

PROTEIN OXIDATION AND PROTEIN NITRATION INFLUENCED BY SODIUM NITRITE IN TWO MEAT MODEL SYSTEMS

ABSTRACT

This study focuses on the effect of $NaNO_2$ on induced protein oxidation in isolated pig myofibrillar proteins suspensions (20 mg/ml) and in two batches of raw porcine patties produced from the *longissimus* muscle of two different pigs. In addition, the possible use of 3-nitrotyrosine as a specific marker for reactive nitrogen species mediated nitration in processed muscle foods was investigated. In the myofibrillar protein isolate, higher protein carbonyl concentrations were found in the $NaNO_2$ treated samples (100 and 1000 mg/kg myofibrillar proteins) immediately after addition of oxidants and $NaNO_2$, suggesting an initial pro-oxidative effect of $NaNO_2$. Thiol compounds decreased rapidly but no clear effect of $NaNO_2$ was observed. No effect of $NaNO_2$ was observed at later time points of induced oxidation at 37°C. Conversely, $NaNO_2$ exhibited antioxidant activity against protein carbonyl formation in raw porcine patties at a dose of 200 mg/kg muscle, but not at 20 mg/kg muscle. However, this antioxidant effect was only seen in patties from one animal, while no effect was seen in the patties of the other animal. 3-Nitrotyrosine, a good marker for oxidative stress *in vivo*, was present in all samples, which was assessed by Western blot and spectrophotometric analyses, but no clear effect of $NaNO_2$ addition or oxidation time was observed on this protein modification.

INTRODUCTION

Nowadays, protein oxidation in meat products receives increasing research interest. Protein oxidation is defined as the covalent modification of a protein induced either by the direct reactions with reactive oxygen and nitrogen species or indirect reactions with secondary oxidation products. As many other macromolecules, myofibrillar proteins are susceptible to oxidative reactions with myosin being the most sensitive (Lund et al., 2011). Oxidative modifications of proteins can change their physical and chemical properties, which has impact on fresh meat quality and the properties for processing (Zhang et al., 2012). Consequently, there is growing interest on antioxidants to inhibit protein oxidation. Mainly phenolic compounds are investigated for this purpose, but also other antioxidants such as carotenoids and tocopherols have shown an antioxidant effect against protein oxidation (Ventanas et al., 2006; Vossen et al., 2012b; Jongberg et al., 2013). Strangely, nitrite has scarcely been investigated for its antioxidant capacity against protein oxidation, although it is commonly used when curing meat and it has several properties affecting proteins (Vossen et al., 2012a; Villaverde et al., 2014). Four different mechanisms have been proposed for the antioxidant effect of nitrite in meat (Arendt et al., 1997): it forms a stable complex by coordination to the iron center of haem proteins and thereby prevents catalytic breakdown of hydroperoxides by the haem proteins; it chelates trace metals which might be possible pro-oxidants; it reacts with meat constituents forming nitroso and nitrosyl compounds, which possess antioxidant activity and it stabilizes the lipid fraction by reaction with the carbon-carbon double bonds, as the amount of reacted nitrite increases with the number of double bonds in lipid model systems.

Several biomarkers, such as the formation of protein carbonyls, loss of thiol groups, protein fragmentation and aggregation, are commonly used to quantify or characterize protein oxidation processes in muscle foods (Lund et al., 2011). As the generation of carbonyl derivatives is orders of magnitude greater than other kinds of protein oxidation, the carbonyl content of proteins has

become the most generally used method for estimation of protein oxidation (Stadtman & Levine, 2000). However, for this study, utilization of a more specific marker that evaluates the role of $NaNO_2$ in the oxidative stability of food products was desirable. In this respect, 3-nitrotyrosine could be an interesting marker. After exposure to reactive nitrogen species, like peroxynitrite, 3-nitrotyrosine is formed and it has extensively been used as marker for nitroxidative stress *in vivo* (Souza et al., 2008). Altered 3-nitrotyrosine concentrations were previously found in oxidatively modified chicken muscles (Stagsted et al., 2004) and isolated myofibrillar porcine muscles treated with sodium nitrite (Villaverde et al., 2014), but its usefulness as a marker in cured and uncured processed meats during shelf life remains unknown.

The isolation of myofibrillar protein is a widely applied technique to investigate meat proteins *in vitro* without the interference of other meat compounds (Park et al., 2006; Estévez & Heinonen, 2010). However, for oxidation processes the importance of the matrix can not be ignored. In addition to the examination of isolated myofibrillar proteins, a more complex meat model system should be considered.

The objective of this study was to investigate the effect of $NaNO_2$ on induced protein oxidation in isolated pig myofibrillar proteins and in raw porcine patties during illuminated chilled display. In addition, the potential use of 3-nitrotyrosine as a specific marker for protein oxidation in combination with $NaNO_2$ will be explored.

MATERIALS AND METHODS

1. Experimental set-up

Myofibrillar protein isolate (MPI)

Three porcine *longissimus* muscles were purchased from a local slaughterhouse (2 days *post mortem*). The muscles were trimmed from visible fat and connective tissue and subsequently

diced into approximately 5 g pieces. The muscle dices of all three animals were mixed well by hand and subsequently vacuum packed and stored at -80°C until use.

Myofibrils were isolated according to Park et al. (2006). Thawed and minced muscle was homogenised by blending 30 s in an ultraturrax with four volumes (w/v) of a cold isolation buffer (10 mM sodium phosphate buffer, 0.1 M NaCl, 2 mM $MgCl_2$ and 1 mM EGTA, pH 7.0). The muscle homogenate was centrifuged at 2000 g for 15 min, and the supernatant was discarded. The pellet was washed two more times with four volumes of the same isolation buffer using the same blending and centrifugation conditions as indicated above. The myofibril pellet was then washed three more times with four volumes of 0.1 M NaCl under the same conditions as above except that in the last wash, the myofibril suspension was filtered through a strainer to remove connective tissue, and its pH was adjusted to 6.0 with 0.1 N HCl prior to centrifugation. The MPI was stored in a tightly capped bottle, kept on ice, and used within 24 h. The protein concentration of the myofibril pellet was measured by the Biuret method (Gornall et al., 1949) using bovine serum albumin as standard.

The MPI was oxidized under meat processing conditions according to Estévez et al. (2009). Briefly, MPI was suspended in 15 mM piperazine-N,N bis(2-ethane sulfonic acid) (PIPES) buffer (pH 6.0) containing 0.6 M NaCl (total volume: 30 ml; 20 mg protein/ml). The MPI suspensions were oxidized with 0.01 mM $FeCl_3$, 0.1 mM ascorbic acid and 1 mM H_2O_2. Three doses of $NaNO_2$ (0, 100 or 1000 mg $NaNO_2$/kg protein) were added and the samples were incubated at 37°C for seven days while constantly stirring. After 30 min (without incubation at 37°C), and 4 and 7 days, suspensions were divided in aliquots of 1.2 ml and stored at -80 °C until analysis. MPI suspended in PIPES buffer without oxidants or $NaNO_2$ was used as non-oxidized control.

Raw porcine patties

Two lean porcine *longissimus* muscles from different animals were purchased from a local slaughterhouse (2 days post mortem). Two separated batches from the two muscles were made and are referred to as "patties A" and "patties B". Three treatments were considered: 0, 20 or 200 mg $NaNO_2$ per kg patty. In the basic formulation, the ingredients per batch were as follows: 500 g of meat, 35 g of distilled water, 8.5 g of sodium chloride and 0, 0.01 or 0.1 g $NaNO_2$. This resulted respectively in patties with 0, 20 or 200 mg/kg $NaNO_2$ or respectively 0, 100 and 1000 mg/kg protein taking into account that the muscles consists of approximately 20% proteins. The muscles were trimmed from visible fat and connective tissue and subsequently diced into approximately 5 g pieces. The meat was ground in a grinder (Omega T-12) equipped with a 10 mm plate. NaCl and $NaNO_2$ were dissolved in the distilled water and immediately added to the meat. After mixing the batter by hand, the mixture was grounded once more with a 3.5 mm plate. Nine patties of 40 g were made per treatment and per batch. Three patties were immediately vacuum packed and stored at -80°C (day 0) and the six remaining patties were wrapped in an oxygen permeable film and subjected to illuminated chilled display (4°C, 1000 lux). After 4 and 7 days of display, three patties per treatment were vacuum packed and stored at -80°C until analysis.

2. Chemical analyses

Thiol concentration of the MPI suspensions

The thiol concentration was determined in the MPI samples after derivatisation by Ellman's reagent, 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) adopted from Jongberg et al. (2013) An aliquot of 1.2 ml MPI suspension was defrosted and centrifuged for 20 min at 2000 g. The supernatant was carefully discarded and 1.0 ml of 0.1 M tris(hydroxymethyl)-aminomethane (TRIS) buffer (pH 8) was added to the pellet, vortexed and centrifuged at 2000 g for 20 min.

After discarding the supernatant, 1.0 ml of TRIS buffer was added to the pellet and everything was transferred to a test tube. The microtube was rinsed twice with 1 ml TRIS buffer, which was also transferred to the test tube. Three ml of 10.0 % sodium dodecyl sulphate (SDS) in 0.1 M TRIS buffer (pH 8.0) was added and the proteins were dissolved by one hour of incubation in a water bath heated to 80°C. After cooling, samples were centrifuged at 1400 g for 10 minutes and the thiol concentration in the supernatant was analyzed. Two ml of 0.1M TRIS buffer (pH 8) and 0.5 ml of 10 mM DTNB dissolved in 0.10 M TRIS buffer (pH 8.0) was added to 0.5 ml supernatant. For each sample a blank was included containing 0.5 ml supernatant and 2.5 ml 0.10 M TRIS buffer (pH 8.0). A solution containing 0.5 ml 5.0 % SDS in TRIS buffer (pH 8.0), 0.5 ml 10 mM DTNB and 2.0 ml 0.1 M TRIS buffer (pH 8) was used as reagent blank. All mixtures were protected against light and allowed to react for exactly 30 minutes. The absorbance was measured spectrophotometrically at 412 nm and the thiol concentration was calculated using the formula of Lambert-Beer ($\varepsilon_{412} = 14000$ M^{-1} cm^{-1}) and expressed in nmol thiol/mg protein. The protein concentrations of the extracts were calcultated from the sample blanks, which were determined spectrophotometrically at 280 nm using a BSA standard curve.

Protein carbonyl content

The protein carbonyl content of the MPI samples and patties was determined by derivatization with DNPH (2,4-dinitrophenyl hydrazine) as described by Levine, Williams, Stadtman, & Shacter (1994) with some modifications. An aliquot of 1.2 ml MPI suspension was defrosted and centrifuged for 20 min at 2000 g. The pellet was then re-suspended in 1 ml phosphate buffer (20 mM, pH 6.5) and four aliquots of 0.2 ml were taken. For the raw patties, 3 g of meat with 30 ml of phosphate buffer (20 mM, pH 6.5 containing 0.6M NaCl) was homogenized and four aliquots

of 0.2 ml were treated with 1 ml icecold TCA (10%) to precipitate the proteins. The samples were left for 15 min in an ice bath and after centrifugation at 2000 g for 30 min, the supernatant was discarded. Another milliliter of icecold TCA (10%) was added and the above mentioned procedure was repeated. For both the MPI suspensions and the patty pellets, two aliquots were treated with 0.5 ml 10 mM DNPH dissolved in 2.0 M HCl and two aliquots were treated with 0.5 ml 2.0 M HCl (blank). The samples were placed on a vortex (350 rpm) for 1h covered from light to derivatise. Subsequently, 0.5 ml icecold 20% TCA was added, vortexed and placed on ice for 15 minutes before centrifugation at 2000 g for 20 min after which the supernatant was discarded. Excess DNPH was removed by washing three times with 1.0 ml of ethanol:ethylacetate (1:1 v/v), vortexing and centrifuging at 2000 g for 20 minutes. After every wash the supernatant was discarded. Following the final wash excess solvent was removed by leaving the samples for 15 min under the hood. The pellets were dissolved in 1.0 ml 6.0 M guanidine hydrochloride in 20 mM phosphate buffer (pH 6.5) and placed on a vortex (350 rpm) for 30 min covered from light. The final solution was centrifuged at 9500 g for 10 minutes to remove insoluble material. The carbonyl concentration (nmol/mg protein) was calculated from the absorbance at 280 nm and 370 nm of the samples using the equation below (Levine et al., 1994).

$$\frac{C_{hydrazone}}{C_{protein}} = \frac{A_{370}}{\varepsilon_{hydrazone,370} \times (A_{280} - A_{370} \times 0.43)} \times 10^6$$

Where $\varepsilon_{hydrazone,370}$ is 22000 $M^{-1} cm^{-1}$ and the carbonyl concentrations obtained from the blanks were subtracted from the contribution obtained from the corresponding treated sample.

3-Nitrotyrosine

3-Nitrotyrosine and actin were qualitatively detected by western immunoblotting in MPI suspensions (n=1) using respectively a mouse monoclonal anti-nitrotyrosine antibody (Abcam, UK, 1:500 dilution) and rabbit anti-actin antibody (Sigma-Aldrich, Belgium, 1:2000 dilution) as primary antibody and IRDye 800 goat anti-mouse IgG (Li-COR, USA, 1:5000 dilution) and IRDye 680 Goat anti-Rabbit IgG (Li-COR, USA, 1:5000 dilution) as secondary antibodies. Gels were scanned and band intensities were measured with an Odyssey infrared fluorescence detection system.

In addition, 3-nitrotyrosine was quantified spectrophotometrically after alkalinisation in both the MPI suspensions and patties according to Fontana et al. (2012) with some modifications.

Aliquots of 1.2 ml MPI suspensions were defrosted and two aliquots of 0.5 ml were taken. After centrifugation at 2000 g for 30 min, the supernatant was carefully discarded. For the raw patties, 3 g of meat with 30 ml of phosphate buffer (20 mM, pH 6.5 containing 0.6M NaCl) was homogenized and two aliquots of 0.2 ml were treated with 1 ml icecold TCA (10%). The samples were left for 15 min in an ice bath and after centrifugation at 2000 g for 30 min, the supernatant was discarded. Another milliliter of icecold TCA (10%) was added and the above mentioned procedure was repeated. To both the MPI and patty pellets 1 ml guanidine-HCl (6M) was added. After one hour on a vortex, 0.6 ml was transferred into a microtube, an equal amount of bicarbonate buffer (0.2 M, pH 10.5) was added and the samples were centrifuged at 9500 g for 10 min. The 3-nitrotyrosine content was quantified spectrophotometrically at 430 nm, using 3-nitro-L-tyrosine as standard. The total protein content of the pellet was quantified at 280 nm using BSA as standard. According to Yang et al. (2010), tyrosine and nitrotyrosine both exhibit an absorption peak at 280 nm, and nitrotyrosine has an additional peak at 430 nm in basic solutions (pH ≥9.5). Results were expressed as nmol 3-nitrotyrosine/mg protein.

Statistical analysis

Statistical analysis was performed using SPSS 22.0. Results were analysed by ANOVA with oxidation time and $NaNO_2$ concentration as fixed effects. The interaction term time $\times$ $NaNO_2$ was excluded from the model when not significant (P>0.05). When the interaction term was found significant, a new factor called "experimental unit" was computed combining oxidation time and $NaNO_2$ concentration and one-way ANOVA considering "experimental unit" as fixed effect was conducted. Mean differences were tested using Tukey's post-hoc test operating at a 5% level of significance.

RESULTS

MPI

Results concerning the oxidized MPI samples are shown in Table 4.1. From the moment the oxidants were added, the thiol concentrations decreased 5-fold compared to the untreated MPI. During the following 7 days a further decrease of thiol groups was noticed (P<0.05), however this decrease was not as pronounced compared to the initial decrease. The addition of sodium nitrite did not affect the thiol content (P>0.05).

Induced *in vitro* oxidation of MPI resulted in up to ten fold higher content of carbonyl compounds when compared to the untreated MPI. During the following 7 days, an overall increase of protein carbonyl compounds (P<0.05) was observed. Also, the addition of sodium nitrite affected the formation of carbonyl compounds as on average higher carbonyl concentrations were found in the oxidized MPI samples treated with 1000 mg/kg $NaNO_2$ compared to the average carbonyls content of the samples without $NaNO_2$ (P<0.05), with intermediate values for the samples treated with 100 mg/kg. This effect resulted mainly from the two fold higher carbonyl concentrations after 30 min of induced oxidation in the nitrite treated samples, while at day 4 and day 7 similar values were found among treatments.

Table 4.1. Effect of nitrite dose on protein thiol, carbonyl and 3-nitrotyrosine content (nmol/mg protein) in myofibrillar protein isolates (MPI) after induced oxidation for 7 days

	Time	NaNO$_2$ (mg/kg) 0	100	1000	Mean
Thiol groups[1]	**MPI***	*51.0±4.2*			
	30 min	20.0±18.3	12.9±4.9	12.0±5.4	14.9^a
	4 days	12.2±1.7	5.74±4.70	6.91±5.61	7.4^b
	7 days	9.84±1.02	11.8±0.49	7.87±0.78	9.6^{ab}
	Mean	15.1	9.4	8.8	
Carbonyl compounds[2]	**MPI***	*1.42±0.46*			
	30 min	3.91±1.38	7.38±1.33	8.57±2.62	6.5^c
	4 days	11.3±3.1	11.0±0.6	11.3±1.86	11.2^b
	7 days	13.2±1.2	13.8±0.9	14.3±2.6	13.8^a
	Mean	9.0^y	10.7^{xy}	11.2^x	
3-Nitrotyrosine[3]	**MPI***	*3.31±0.48*			
	30 min	3.28±0.71^x	1.51±0.50^{b,y}	1.31±0.17^{b,y}	2.1
	4 days	3.11±0.69	3.26±0.66^a	3.55±1.16^a	3.3
	7 days	3.23±0.08	3.02±0.52^{ab}	3.33±0.60^a	3.2
	Mean	3.2	2.5	2.8	

*MPI: the non-oxidized myofibrillar proteins isolates, suspended in a PIPES buffer without pro-oxidants and NaNO$_2$, was not included in the statistical model;

[1]$p_{nitrite}$ =0.174; p_{time} = 0.044; $p_{nitrite \times time}$ = not significant;

[2]$p_{nitrite}$ =0.037; p_{time} < 0.001; $p_{nitrite \times time}$= not significant;

[3]$p_{nitrite}$ = 0.105; p_{time} <0.001; $p_{nitrite \times time}$ = 0.015;

$^{a-c}$ Effect of oxidation time: values with a different letter within a column are different (P<0.05);

$^{w-z}$ Effect of NaNO$_2$: values with a different letter within a row are different (P<0.05).

Immunoblotting using an antibody against 3-nitrotyrosine was performed to investigate the occurrence of 3-nitrotyrosine in the oxidized MPI. Figure 1 confirms the presence of 3-nitrotyrosine in all samples. A spectrophotometric method was used to quantify 3-nitrotyrosine. The 3-nitrotyrosine concentrations (Table 4.1) remained constant during the experiment, except for two fold lower 3-nitrotyrosine concentrations in samples treated with 100 and 1000 mg/kg after 30 min of induced oxidation. Interestingly, although only measured in one sample, also two fold lower 3-nitrotyrosine/actin band intensity ratios were observed after 30 min of induced oxidation on the Western Blot in samples treated with 100 and 1000 mg/mg sodium nitrite (data not shown).
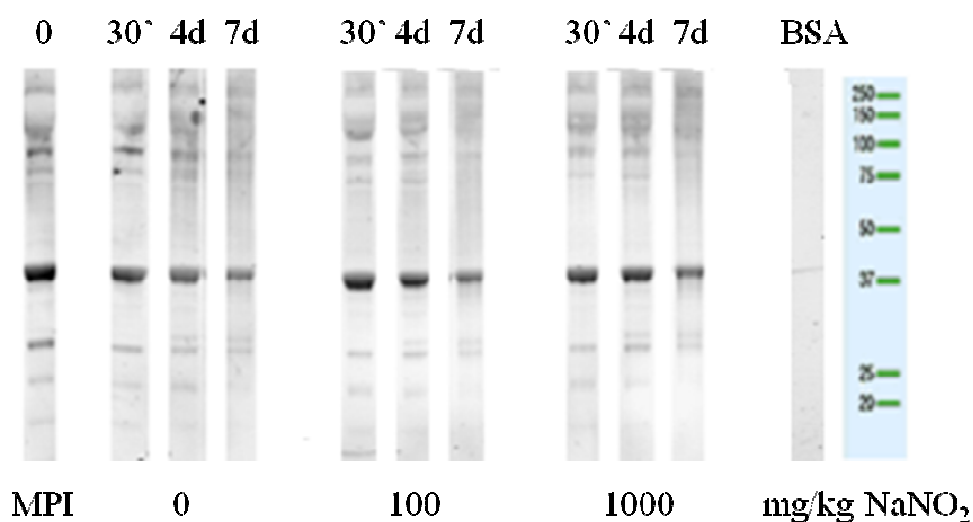
Figure 1. Representative Western blot for 3-nitrotyrosine during induced *in vitro* oxidation of myofibrillar protein isolates with different $NaNO_2$ levels. MPI: non-oxidized myofibrillar proteins isolate, suspended in a PIPES buffer without pro-oxidants and $NaNO_2$. BSA: bovine serum albumin, negative control.

Patties

The results of patties A and patties B are shown separately (Table 4.2) as a significant effect of animal was observed on 3-nitrotyrosine ($P<0.05$), but not on carbonyl content ($P>0.05$). In addition, the effect of $NaNO_2$ and days of display on the carbonyl and 3-nitrotyrosine content differed between the two animals.

The carbonyl concentrations in patties A were not affected by the added sodium nitrite ($P>0.05$), nor by the display period ($P>0.05$). Higher 3-nitrotyrosine concentrations were found across days of display in patties treated with 20 mg/kg $NaNO_2$ compared to the 200 mg/kg treatment ($P<0.05$), with intermediate values for untreated patties. No significant changes occurred during illuminated chill display ($P>0.05$).

For patties B, lower carbonyl concentrations were found across days of display in patties treated with 200 mg/kg sodium nitrite compared to 0 and 20 mg/kg ($P<0.05$). The carbonyl concentrations of the patties did not change significantly during the whole period of illuminated chilled display ($P>0.05$). The 3-nitrotyrosine content was up to 1.5 fold lower compared to the concentrations in the patties A, but was not affected by the added sodium nitrite content ($P>0.05$), nor by the display period ($P>0.05$).

Table 4.2. Effect of nitrite dose on protein carbonyl and 3-nitrotyrosine content (nmol/mg protein) in raw patties A and B (n=3) after illuminated chilled display for 7 days

Time	$NaNO_2$ (mg/kg) 0	20	200	Mean
Carbonyl compounds				
Patties A[1]				
0 days	2.72±1.03	4.04±0.83	3.32±0.84	3.30
4 days	3.64±0.32	3.96±1.60	3.15±0.78	3.54
7 days	3.99±1.37	4.32±2.16	2.31±0.17	3.66
Mean	3.43	4.11	3.03	
Patties B[2]				
0 days	4.72±0.99	4.50±1.77	1.99±0.35	3.94
4 days	4.52±0.84	3.51±1.54	2.74±0.43	3.58
7 days	3.78±0.81	2.58±0.61	2.61±0.54	3.06
Mean	4.32[a]	3.69[a]	2.44[b]	
3-Nitrotyrosine				
Patties A[3]				
0 days	7.54±0.95	8.59±1.00	6.57±0.55	7.57
4 days	7.11±0.47	8.75±1.52	6.30±0.44	7.39
7 days	6.99±0.47	7.41±1.53	6.42±0.76	6.93
Mean	7.21[ab]	8.25[a]	6.43[b]	
Patties B[4]				
0 days	5.58±1.19	5.62±1.80	6.26±1.51	5.80
4 days	5.10±0.24	5.69±1.16	5.13±0.68	5.38
7 days	4.55±0.32	5.54±1.72	5.20±0.52	5.15
Mean	5.12	5.63	5.50	

[1] $p_{nitrite} = 0.115$; $p_{time} = 0.892$; $p_{nitrite \times time}$ = not significant;

[2] $p_{nitrite} = 0.003$; $p_{time} = 0.209$; $p_{nitrite \times time}$ = not significant;

[3] $p_{nitrite} = 0.001$; $p_{time} = 0.331$; $p_{nitrite \times time}$ = not significant;

[4] $p_{nitrite} = 0.468$; $p_{time} = 0.341$; $p_{nitrite \times time}$ = not significant;

[a-c] Effect of oxidation time: values with a different letter within a column are different (P<0.05);

[w-z] Effect of $NaNO_2$: values with a different letter within a row are different (P<0.05).

DISCUSSION

This study investigates the effect of sodium nitrite on protein oxidation and nitration in two different meat model systems.

$NaNO_2$ has antioxidant activity against lipid oxidation, which is mostly explained by its ability to break the radical chain processes after its conversion to $NO\bullet$ and by chelating iron, a known oxidation promoter (Skibsted, 2011). As the mechanism behind the oxidation of proteins is believed to proceed via a free radical chain reaction similar to that of lipid oxidation (Lund et al., 2011), it was expected that $NaNO_2$ would have an antioxidant effect against protein oxidation. Yet, this positive effect was only found in the raw patties from one animal at a dose of 200 mg/kg muscle.

On the other hand, $NaNO_2$ could also act as pro-oxidant. After its reduction to $NO\bullet$, peroxynitrite ($ONOO^-$) can be formed by reacting with $O_2\bullet^-$. Peroxynitrite can induce lipid oxidation in food systems (Brannan et al., 2001), protein oxidation *in vivo* (Tiago et al., 2008) and can cause discolouration of muscle food by oxidizing oxymyoglobin (Connolly & Decker, 2004). According to Rubbo et al. (1994), the pro-oxidant versus antioxidant outcome critically depends on the relative concentrations of individual reactive species such as $O_2\bullet^-$, H_2O_2 and $OH\bullet$. These reactive species are abundantly present in *in vitro* oxidation systems, which could explain the pro-oxidative effect of sodium nitrite in the MPI samples, half an hour after the addition of the oxidants. Maybe the intensity of the induced oxidation masked this effect at later time points. Also, sodium nitrite and the pro-oxidants were added simultaneously, which could have affected the reaction pathways of the nitrite. In the porcine patties sodium nitrite can firstly react with the meat components before oxidation during shelf life occurs, possibly explaining why a different effect of nitrite was found in the myofibrillar isolates compared to the patties. For example, nitrite reacts with myoglobin resulting in nitrosylmyoglobin (Sullivan & Sebranek, 2012), which appears to be active as an antioxidant both through dissociation of nitric oxide and through direct

reaction with activated oxygen species (Skibsted, 2011). Likewise, nitric oxide reacts with the free radical intermediates of lipid oxidation and these compounds can function both as nitric oxide donors and antioxidants (Nicolescu et al., 2004). We did not include measurements after 30 min of oxidation for the patties as the oxidation processes during chilled display were less intense compared to induced oxidation with pro-oxidans and no effect at that time point was expected. Villaverde et al. (2014) reported a negligible effect of sodium nitrite on α-aminoadepic semialdehyde (a specific protein carbonyl compound) after 4 days of induced oxidation in a similar *in vitro* MPI system, which is in accordance with the present results.

The oxidation of thiol groups leads to a series of complex reactions resulting in the formation of various oxidized products such as sulfenic acid, sulfinic acid and disulfide cross links (Lund et al., 2011). In addition, Sullivan & Sebranek (2012) found decreasing thiol groups with increasing ingoing sodium nitrite, and dedicated this to the formation of S-nitrosothiol groups. A lower thiol content was therefore expected in the sodium nitrite treated samples, but it is likely that the intensity of the induced oxidation, which resulted in an overall large loss of thiol groups, masked this effect. The values obtained for the loss in thiol groups and progress of protein carbonyl formation in induced *in vitro* oxidation of MPI compare well with the observations of respectively Liu & Xiong (2000) and Estévez & Heinonen (2010). The effects are greater compared to those usually found in real meat products, as the concentrations of oxidants applied in a model system are designed to stimulate oxidation.

It is not clear whether the addition of sodium nitrite affected the 3-nitrotyrosine content *post mortem* and additional investigations are necessary to explore the occurrence of 3-nitrotyrosine in muscle foods and its potential influence on the quality. An increase in 3-nitrotyrosine content in the sodium nitrite treated samples was somehow expected, but on the contrary, even a decrease was seen after 30 min of induced oxidation in myofibrillar protein isolates. From this observation we would conclude that 3-nitrotyrosine is probably not a good marker for protein oxidation in

cured meat products, but conversely, Villaverde et al. (2014) did find increased concentrations in myofibrillar protein isolates treated with sodium nitrite. However they added 19 and 38 fold higher amounts of sodium nitrite compared to our study. One item to be further explored is the variation between animals and between muscles of the basal 3-nitrityosine content. The occurrence of 3-nitrotyrsosine in muscle proteins is a result of oxidative stress *in vivo* (Tiago et al., 2008), so basal levels of 3-nitrotyrosine in the porcine patties as well as in the myofibrillar protein isolates were expected. To my knowledge this is the first time that 3-nitrotyrosine was quantified per milligram of meat protein, although Stagsted et al. (2004) did find 3-nitrotyrosine in chicken meat using Western Blot. Stagsted et al. (2004) found significantly higher basal concentrations of 3-nitrotyrosine in meat of chickens fed a low antioxidant diet compared to chickens fed a low antioxidant diet in combination with corn. These authors suggested that the nitration of actin could have possible implications on the texture and water holding capacity of fresh meat. As 3-nitrotyrosine can affect a protein's structure and function (Kuo et al., 2000), it was hypothesized that 3-nitrotyrosing could be a marker for protein oxidation in cured meat products affecting the quality of muscle foods. From the present results it can be concluded that nitrite-curing has no effect on 3-nitrotyrosine *post mortem*, implying that it is not a good marker for this purpose, but this has to be confirmed in further research. Other compounds originating from the reaction of nitrite with proteins, such as nitrosotryptophan and S-nitrosocysteine, are reported, but it should be investigated whether these compounds are relevant markers for the effect of reactive nitrogen species on the quality of meat products.

CONCLUSIONS

The present study demonstrates the importance of individual animal and matrix effects when investigating protein oxidation in meat systems. Sodium nitrite showed pro-oxidative activities in isolated myofibrillar proteins, but antioxidant activities in raw porcine patties. It would therefore

be interesting to investigate the effect of nitrite in cooked pork patties as nitrite is abundantly used in cooked meat products. 3-Nitrotyrosine is abundantly present in untreated myofibrillar proteins, but whether other compounds, such as antioxidants, can influence its occurrence *post mortem* and whether this is a good marker for protein oxidation in processed meats, remains to be elucidated.

ACKNOWLEDGEMENTS

ENRICHMENT OF MEAT WITH N-3 POLYUNSATURATED FATTY ACIDS

Does the efficacy to increase the n-3 PUFA concentration of meat products depend on the n-3 PUFA source in the feed?

Part I Lowering the ingoing nitrite dose	Part II Increasing the n-3 PUFA content
CH1 With a dog rose extract	CH5 Role of n-3 PUFA source
CH2 With sodium ascorbate	CH6 Effect on sensory quality
CH3 With a pre-converted extract	CH7 Role of α-tocopherol in feed
CH4 Nitrite and protein oxidation?	CH8 Effect on health
	CH9 General discussion and future prospects

FATTY ACID COMPOSITION OF FRESH MEAT, SUBCUTANEOUS FAT, DRY FERMENTED SAUSAGE AND DRY CURED HAM INFLUENCED BY LINSEED OIL, FISH OIL OR MICROALGAE INCLUDED IN THE PIG FEED

Partly redrafted after Vossen E., Van Mullem D., Raes K., De Smet S. (2009). Fatty acid profile of meat and fermented sausages influenced by including linseed, fish oil or microalgae in pig feed. Proceedings 55th IComst, Copenhagen, Denmark, 16-21/08/2009 short paper PE9.30

and Vossen E., Van Mullem D., Raes K., De Smet S. (2010). Fatty acid composition and sensory acceptability of dry cured ham influenced by linseed oil, fish oil or microalgae included in the pig feed. Proceedings 56th IComst, Jeju, Korea, 15-20/08/2010, short paper B017.

ABSTRACT

It is now well established that n-3 polyunsaturated fatty acids have a protective influence on several chronic diseases. The objective of the present study was to investigate the fatty acid profile of fresh meat, subcutaneous fat, dry fermented sausages and dry cured ham of pigs fed different n-3 polyunsaturated fatty acid sources. Crossbred pigs were given an experimental diet supplemented with 0.6g/100g linseed oil (LIN), 0.8 g/100g fish oil (FISH) or dried microalgae (ALG) at 0.3, 0.6 or 1.2 g/100 g (ALG LOW, ALG MEDIUM and ALG HIGH respectively). In the control group soybean oil was added to the diet. The fatty acid composition of the samples was analyzed by gas chromatography. Similar results were found for the different products investigated: significantly higher C18:3n-3 (ALA) proportions in the LIN group and higher proportions of C20:5n-3 (EPA) in the FISH group were found compared to all other groups. The C22:6n-3 (DHA) proportions in the FISH group and ALG groups were significantly higher compared to the SOY and LIN group. The DHA and EPA proportions increased with increasing amounts of microalgae in the feed, which was unexpected for EPA, as no EPA was originally present in the ALG diets. Lower proportions of the long chain n-3 and n-6 were found in the subcutaneous fat compared to the fresh meat. Manufacturing processes such as fermentation and long term dry curing did not drastically affect the fatty acid profile of the meat products. The daily recommended intake of EPA and DHA (%RNI) can be increased considerably with these n-3 polyunsaturated fatty acids enriched products, even up to 34% by consuming 50g of dry fermented sausage of the ALG HIGH group.

INTRODUCTION

It is generally accepted that the n-3 polyunsaturated fatty acids (PUFA), α-linolenic acid (ALA; C18:3n-3) and especially its metabolites eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) have a protective influence on several chronic diseases (Ruxton et al., 2004; Narayan et al., 2006; Simopoulos, 1999). Also n-6 FA and in particular linoleic acid (LA, C18:2n-6) and arachidonic acid (AA, C20:4n-6) have important metabolic functions (Johnson & Fritsche, 2012), however, the diets of Western countries do provide sufficient amounts of these n-6 FA. On the contrary, the daily intake of n-3 PUFA does not fulfil the recommendations in most countries. Belgian adolescents and women consume on average 0.07-0.1% EPA+DHA and 0.57-0.64% ALA of their total daily energy intake (%E), while the Superior Health Council of Belgium (2006) recommends that at least 0.3%E should originate from EPA+DHA and 1%E should originate from ALA (Sioen et al., 2006 and Sioen et al., 2007). Despite attempts to provide education about healthier eating patterns, there are several barriers such as a lack of interest towards changing one's diet, or concerns about having to compromise on taste or enjoyment (Kearney & McElhone, 1999). A successful strategy to improve the n-3 PUFA content of the overall diet would be to provide these fatty acids in food products that are already popular. Although meat only partly contributes to the total fat intake of the diet, optimizing its fatty acid profile still deserves attention due to the high meat intake in industrialized countries (Howe et al., 2006). Even though people nowadays consume less meat, it is still one of the principal food components of our diets, so I believe that when several meat products are enriched with n-3 PUFA it can be a possible solution to increase the daily n-3 PUFA intake. According to the human consumption database of Belgium (Devriese et al., 2006), 75% of the Belgian population consumes 5-7 times a week meat. Of course, the enrichment of meat should be only one aspect of a larger strategy for enriching other widely consumed foodstuffs with n-3 PUFA. Besides adding n-3 PUFA sources during the processing of foods or

administering supplemental oils and capsules, incorporation of n-3 PUFA rich products like grass, rapeseed, linseed and fish oil in livestock feeds, resulting in accumulation of these fatty acids in animal products, received a lot of interest (Wood et al., 2008; Raes et al., 2004). This approach is of particular interest, since fatty acids provided through this route are thought to be more stable to the effects of processing than are fats and oils added to foods during manufacturing (Williams, 2000). In addition, Deckelbaum et al. (2008) stated that habitual consumption of n-3 PUFA may be more beneficial than short-term consumption.

As the conversion of ALA to EPA is low (<10%) and further conversion to DHA is even worse (Hussein et al., 2005), the direct supply of EPA and DHA in animal feeds instead of ALA-rich sources such as linseed and rapeseed is considered to be more efficient for increasing the long-chain n-3 PUFA content of animal products. Generally, fish oil or fish meal is used as a direct source of EPA and DHA in feeds, but caution is needed as many species have been fished almost to extinction and we are on course to eliminate the world's supply. Brunner et al. (2009) concluded that urgent national and international action is necessary to address the tensions arising from increasing human demand for fish and seafood, and rapidly declining marine ecosystem health. Given that microalgae are the original source of EPA and DHA in the marine food chain, dried marine algae have also been included in animal feeds (Nieto & Ros, 2012). However, studies including marine algae focus mainly on poultry (Rymer et al., 2010), while experiments conducted on pig and its meat products are rather limited (Marriott et al., 2002; Sardi et al., 2006; Sárraga et al., 2007; Meadus et al., 2009) and our present knowledge of animal response to dietary microalgae is relatively scanty.

This research was performed to evaluate the incorporation of n-3 PUFA in meat and subcutaneous fat through different n-3 PUFA sources in pig feed. As only a small part of pork is consumed as fresh meat cuts, investigating the manufacturing of meat products enriched in n-3 PUFA through pig feed is of interest. Dry fermented sausages and long term dry cured hams

were produced to assess the effect of manufacturing conditions on the fatty acid profile of these products. Special attention was paid to the use of microalgae as a sustainable source for long chain n-3 PUFA.

MATERIALS AND METHODS

1. Experimental set-up and sampling

Six groups of ten female crossbred pigs each were fattened under commercial conditions on different diets from 75 kg until 110 kg live weight. Water and feed was offered *ad libitum*. The six experimental diets consisted of different sources of n-3 PUFA: 0.8 g/100 g feed linseed oil (LIN group, rich in ALA), 1.3 g/100 g feed fish oil (FISH group, rich in EPA and DHA) or dried microalgae (ALG group, rich in DHA). Three ALG groups were considered: ALG LOW, ALG MEDIUM and ALG HIGH with respectively 0.3, 0.6 and 1.2 g dried microalgae per 100 g feed. In the control group (SOY group) soybean oil was included in the feed as n-6 PUFA source. Linseed oil was added to the ALG diets to obtain equal amounts of n-3 PUFA in the LIN, FISH and ALG diets. As the n-6 PUFA content was also kept constant, equal ratios of n-6/n-3 were provided through those diets. All diets were based on barley, wheat and soybean meal and manufactured by Lambers-Seghers (Baasrode, Belgium). The total PUFA content was kept constant and the total fat content was similar for all groups (between 4.3 and 4.8 %, as fed) by the addition of rendered animal fat or coconut fat. All diets were formulated to an equal energy supply (2230 kcal/kg, as fed) and supplemented with 150 mg/kg α-tocopheryl acetate and 0.4 mg/kg organic selenium (Selplex, Alltech, Deinze, Belgium). The fish oil was supplied by INVE Technologies NV (Dendermonde, Belgium), the freeze-dried *Schizochytrium* microalgae from Martek Biosciences Corp. (Martek DHA gold®, Colombia, MD, USA), linseed and other

ingredients were purchased on the local market. The composition of the experimental diets is presented in Table 5.1. Feed samples were collected for fatty acid analysis.

All pigs (ten per treatment) were slaughtered at approximately 110 kg live weight in a commercial slaughterhouse after electrical stunning (Westvlees, Westrozebeke, Belgium). After slaughtering and cooling for 24h, fresh meat samples were obtained from M. *longissimus dorsi* (slices of 2.5 cm) and subcutaneous fat was taken from the back-fat. Samples were stored vacuum packed at -20°C until analysis. From three animals also left shoulder meat and additional subcutaneous fat was sampled for the production of dry fermented sausages (a mixed sample was made per group) and the left hams of six animals per group were sampled for the production of dry cured hams.

Table 5.1. Composition of the experiment diets (g/100g feed, as fed)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high
Corn	10.0	7.0	9.0	9.0	9.0	9.0
Wheat gluten	8.0	7.0	7.8	10.0	10.0	10.0
Barley	15.0	16.5	15.0	15.0	15.0	15.0
Wheat	16.9	17.0	16.0	15.0	15.0	15.0
Wheat shorts	10.0	9.1	11.0	10.0	10.0	10.0
Sorghum	10.0	10.0	10.0	10.0	10.0	10.0
Soybean meal 47% CP	8.8	8.9	8.0	6.8	6.7	6.6
Maniok	6.0	7.0	6.0	5.0	5.0	5.0
Peas	3.0	4.0	3.5	3.0	3.0	3.0
Rapeseed scrap	3.0	4.0	4.0	6.0	6.0	6.0
Palm kernel shell	3.6	4.0	4.0	3.9	3.7	3.4
Rendered Animal Fat	2.0	1.7	1.45	-	-	-
Coconut oil	-	-	-	2.40	2.44	2.48
L-Threonine	0.08	0.07	0.08	0.07	0.07	0.07
DL-Methionine	0.02	0.01	0.02	0.01	0.01	0.01
L-Lysine	1.05	0.91	0.99	1.03	1.03	1.04
Ca carbonate 38%	1.1	1.0	1.1	1.0	1.0	0.93
Vitamin-mineral premix[1]	1.0	1.0	1.0	1.0	1.0	1.0
α-tocopheryl acetate[2]	0.20	0.20	0.20	0.20	0.20	0.20
Soybean oil	0.38	-	-	-	-	-
Linseed oil	-	0.55	-	0.31	0.21	-
Fish oil	-	-	0.83	-	-	-
Dried microalgae	-	-	-	0.30	0.60	1.22
Energy (kcal/kg, as fed)	2250	2237	2233	2225	2225	2225

[1]Vitamin-mineral premix contained 5 g/kg α-tocopheryl acetate and 0.04 g/kg organic selenium (Selplex, Alltech, Dienze, Belgium);
[2]α-tocopheryl acetate concentrate contained 50 g/kg α-tocopheryl acetate.

2. Carcass measurements

Warm carcass weight and carcass lean meat percentage was assessed at the slaughterhouse for all pigs (n=60). At 24h *post mortem*, pH (Knick portamess 654, Knick Elektronische Messgeräte GmbH, Berlin, Germany) with Schott N5800A electrode (Schott Instruments, Mainz, Germany) and conductivity (Pork Quality Meter, Kombi, Intek GmbH, Aichach, Germany) were measured in the ham (M. s*emimembranosus*) and in the loin (M. *longissimus dorsi*).

3. Production of dry fermented sausage and dry cured ham

Dry fermented sausage

Dry fermented sausages were produced at Ter Groene Poorte (Brugge, Belgium) using a standard commercial recipe. For each treatment, a batch was made that consisted of 2.1 kg lean shoulder meat, 0.9 kg subcutaneous fat, 84 g NaCl, 3 g KNO_3, 30 g spices (Raps, Beringen, Belgium) and 1.5 g starter culture (Biosprint from Raps, Beringen Belgium). All sausages were prepared on the same day. Frozen shoulder meat from three different animals was minced in a bowl chopper, together with all additives, except NaCl. Subsequently, the frozen subcutaneous fat was added and mincing was continued until a homogenous batter was reached. Then, NaCl was included and the batter was further chopped until a temperature of -2°C was reached. The mixture was stuffed into 60 mm diameter casings, hand-linked at 30 cm and the sausages were left for six hours at ambient temperature (day 1). After that, the sausages were placed in a fermentation chamber and the following conditions of temperature and relative humidity (RH) were applied: 24°C and 95% RH (day 2), 23°C and 92% RH (day 3), 21°C and 90% RH and smoking for one hour (day 4), 20°C and 88% RH (day 5 until day 7) and from day 8 until day 23 the temperature was kept at 17°C with 76% RH. Finally, the dry fermented sausages were

vacuum packed and stored at 4°C for colour measurements and sensory analysis (See Chapter 6) and at -20°C for chemical analyses.

Dry cured hams

The dry cured hams, containing essentially 3 muscles (M. *semimembranosus,* M. *biceps femoris,* and M. *semitendinosus*), were manufactured at Grega (Buggenhout, Belgium) using a standard commercial protocol under controlled conditions of temperature and relative humidity. The fresh hams were embedded in sea salt and left in a cool room for 1 week. Then, the salt was removed and the hams were salted again with sea salt. The second salting period lasted two weeks. After that, the salt was removed and the hams were allowed to rest for 56 to 70 days under cooled conditions. Subsequently, the hams were washed with water and the surface was covered with a thin layer of fat with spices. Finally, the hams were hung in a drying room for drying and maturation. After 19 months of ripening, the hams were deboned and shipped to the laboratory. The subcutaneous and intramuscular fat was removed and the dry cured hams were subsequently vacuum-packed and stored at 4°C for colour measurements and sensory analysis (see Chapter 6) and at -20°C for chemical analyses (slices of 2.5 cm were stored separately).

4. Proximate composition

Dry matter, crude protein and crude fat content of the fresh meat (n=3), dry fermented sausage (n=2) and dry cured ham (n=6) were determined according to the ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods, respectively. Samples were taken at random from three animals. Analysis was carried out in single and expressed as g/100 g fresh matter. The pH of the dry cured hams (n=6, M. *semimembranosus*) and dry fermented sausages (n=3) were measured per treatment.

5. Fatty acid analysis

The lipids were extracted from the samples using chloroform/methanol (2/1; v/v) (modified after Folch et al., 1957). Fatty acids were methylated according to Raes et al. (2001) and analysed by gas chromatography (HP6890, Brussels, Belgium) on a CP-Sil88 column for FAME (100 m × 0.25 mm × 0.25 µm; Chrompack, The Netherlands). Peaks were identified based on their retention times, corresponding with standards (NuChek Prep., IL, USA; Sigma, Bornem, Belgium). Nonadecanoic acid (C19:0) was used as an internal standard to quantify the individual and total fatty acids. A number of samples were taken at random per treatment, i.e. n=5 for fresh meat, n=6 for subcutaneous fat, n=3 for dry fermented sausage and n=6 for dry cured ham. The fatty acid profiles are expressed in g/100 g of total FAME (fatty acid methyl esters) and the EPA+DHA and total fatty acid content is expressed as g FA/100 g fresh matter.

The EPA+DHA content expressed per 100 kcal was calculated using the mean fat and protein content and the Atwater nutrient conversion factors 9 and 4 kcal/g of fat and protein respectively. The recommended daily intake of EPA+DHA in Belgium is 0.3%E (Superior Health Council of Belgium, 2006) which corresponds to 667 mg EPA+DHA/day taking into account an average daily total energy intake of 2000 kcal. The percentage of the daily recommended nutrient intake for the experimental meat products (%RNI) was calculated assuming a daily intake portion of 50 g product and a recommended daily intake of 667 mg EPA+DHA per day. Finally, it was investigated whether the n-3 PUFA enriched products could be claimed being 'a source of' or 'high in' n-3 PUFA.

'A claim that a food is a *source* of n-3 PUFA, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 0.3 g ALA per 100 g and per 100 kcal, or at least 40 mg of EPA+DHA per 100 g and per 100 kcal. A claim that a food is *high* in n-3 PUFA, and any claim likely to have the same meaning for the consumer, may only be

made where the product contains at least 0.6 g ALA per 100 g and per 100 kcal, or at least 80 mg of EPA+DHA per 100 g and per 100 kcal' (European Commission, 2010).

6. Statistical analysis

The data were submitted to analysis of variance with diet as main effect (SPSS Statistics 22.0). Mean differences between groups were tested using the Tukey post hoc test operating at a 5% level of significance.

RESULTS

1. Carcass characteristics

The mean ($\pm$ standard deviation) warm carcass weight (100 ± 6 kg) and carcass lean meat percentage ($62.6 \pm 2.7\%$) did not differ between feeding groups ($P>0.05$). Likewise, conductivity in the loin (4.80 ± 1.24) and the ham (6.07 ± 2.07) were not influenced by the diet ($P>0.05$), nor was the pH value of the ham (5.57 ± 0.11). The pH of the loin was not different among the feeding groups (overall average 5.60 ± 0.13), except for a lower pH value for DHA MEDIUM group (5.54 ± 0.10) compared to the LIN group (5.70 ± 0.13; $P<0.05$).

2. Proximate composition

For the fresh meat, no effect of diet was found for the mean ($\pm$ standard deviation) dry matter (29.2 ± 0.9 g/100 g meat), fat (1.18 ± 0.63 g/100 g meat) and protein (25.1 ± 0.6 g/100 g meat) content (all $P>0.05$).

The mean dry matter content of the dry fermented sausages was 64.3 ± 1.3 g/100 g sausage and no effect of treatment was found ($P>0.05$). However, for the fat and protein content significant

differences among treatments were found (both P<0.05). The mean fat content of the dry cured sausages was 33.4 ± 1.7 g/100 g sausage, with the highest fat content in the ALG HIGH group (36.1 ± 1.6 g/100 g sausage) and the lowest in the LIN group (31.6 ± 1.2 g/100 g sausage). The mean protein content was 22.7 ± 1.6 g/100 g sausage, with the highest content in the ALG MEDIUM group (25.5 ± 0.7 g/100 g sausage) and the lowest content in ALG HIGH group (21.2 ± 0.58 g/100 g sausage).

For dry cured ham no effect of feeding group was found for the dry matter (46.4 ± 1.18 g/100 g ham), fat (3.46 ± 0.78 g/100 g ham) and protein (28.7 ± 0.96 g/100 g ham) content (all P>0.05). The mean pH of the dry fermented sausages was 5.07 ± 0.06 and 5.66 ± 0.07 for the dry cured hams. Differences between feeding groups were found for both the sausages and hams (P<0.05), however, these differences were limited in magnitude and are not further discussed.

3. Fatty acid composition

Table 5.2 shows the PUFA composition of the feed. In agreement with the trial design, the total PUFA content was similar for all diets, with the main difference between SOY and the other groups being the amount of n-3 PUFA. ALA was the major PUFA in the LIN diet. The FISH diet provided the long chain n-3 PUFA EPA and DHA, whereas the ALG diets provided DHA in different doses.

Table 5.2. Fatty acid composition of the experimental diets (g FA/100g total FA)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high
C18:3n-3	3.51	6.70	2.73	3.45	3.40	2.63
C20:5n-3	<0.10	<0.10	2.19	0.12	0.13	0.25
C22:5n-3	<0.10	<0.10	0.30	<0.10	<0.10	<0.10
C22:6n-3	<0.10	0.19	1.87	1.80	3.62	7.12
C18:2n-6	32.5	24.6	24.4	29.9	28.8	26.7
C20:4n-6	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
PUFA	36.3	31.9	32.2	35.4	36.1	37.0
SFA	29.3	33.8	33.3	43.6	44.4	42.9
MUFA	32.2	32.1	30.9	19.0	16.3	15.2
Total FA (g/100 g feed)	4.34	4.39	4.59	3.60	3.68	4.02

SOY=soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);

SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0;

MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1;

PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3.

The fatty acid composition of the fresh meat is summarized in Table 5.3. The fatty acid composition of the experimental diets is clearly reflected in the fresh meat samples. The LIN diet resulted in a higher ALA proportion compared to all other groups (P<0.05). As expected, the EPA proportion was the highest in the FISH group, with a seven fold higher proportion compared to the SOY group (P<0.05). Relatively high concentrations of EPA were found in the ALG groups, although only low amounts of EPA were present in the ALG experimental feeds. The EPA proportions of the three ALG groups increased with increasing amounts of microalgae in the experimental feeds and moreover, the EPA proportion of ALG HIGH was comparable to the proportion found in the FISH group. The EPA proportion of the LIN group was three fold lower compared to the FISH group and did not differ from the ALG LOW and ALG MEDIUM groups (P>0.05). Still, the EPA proportion of the LIN group was almost three fold higher compared to the SOY group (P<0.05), which indicates a modest but significant conversion of ALA to EPA. As expected, the DHA proportions in the FISH group and ALG groups were higher compared to the SOY and LIN group (P<0.05). The highest DHA proportion was found in the meat of the ALG HIGH group, which was ten fold higher compared to the SOY group (P<0.05). The DHA content in the meat of the ALG groups increased with increasing amounts of microalgae in the experimental feeds and the DHA proportion of the FISH group was intermediate between ALG LOW and ALG MEDIUM. DPA was found in the meat samples of all experimental groups, although it was not present in the diets. The DPA proportion in the fresh meat was two to three fold higher in the LIN and FISH groups compared to the other groups (P<0.05).

The LA proportion was the highest in fresh meat of the LIN group, which was not expected as higher amounts of LA were present in the other diets, especially the SOY diet. The LA proportion of the fresh meat from the LIN group was 1.5 fold higher compared to the ALG LOW group (P<0.05) which had the lowest proportion of LA. AA, the metabolite of LA, was not

affected by the diets, but was considerably high when comparing with the long chain n-3 fatty acids.

Table 5.3 Fatty acid composition of fresh meat (M. *longissimus dorsi,* g FA/100 g total FA; n=5)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
C18:3n-3	0.535[b]	1.38[a]	0.622[b]	0.601[b]	0.506[b]	0.524[b]	0.060	<0.001
C20:5n-3	0.168[d]	0.488[c]	1.30[a]	0.312[cd]	0.704[bc]	1.06[ab]	0.084	<0.001
C22:5n-3	0.557[b]	0.918[a]	0.988[a]	0.395[b]	0.422[b]	0.493[b]	0.049	<0.001
C22:6n-3	0.198[d]	0.452[cd]	1.02[bc]	0.784[cd]	1.69[ab]	2.32[a]	0.148	<0.001
C18:2n-6	11.9[ab]	15.4[a]	11.9[ab]	9.69[b]	12.0[ab]	12.9[b]	0.500	0.024
C20:4n-6	2.96	3.57	2.55	2.39	3.52	3.95	0.182	0.066
PUFA	17.7[ab]	23.8[a]	19.5[ab]	15.3[b]	20.4[ab]	23.0[ab]	0.875	0.030
SFA	33.0	33.7	33.9	36.8	35.0	34.8	0.444	0.179
MUFA	45.0[a]	39.2[ab]	42.9[ab]	44.5[a]	40.3[ab]	36.7[b]	0.798	<0.001
Total FA (g/100 g meat)	1.44	1.18	1.42	1.49	1.28	1.07	0.057	0.197

SOY= soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);

SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0;

MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1;

PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3;

SEM= standard error of the mean calculated from all groups (n=30);

[a-d]Values with different letters in the same row indicate significant differences (P<0.05).

The fatty acid composition of the subcutaneous fat is tabulated in Table 5.4. As for the fresh meat samples, higher (both P<0.05) proportions of ALA and EPA were found in the subcutaneous fat of the LIN and FISH groups respectively, compared to all other groups. The EPA proportion of the ALG HIGH group was again unexpectedly high, however still 2 fold lower than for the FISH group. In contrast to the fresh meat, the EPA proportions in the subcutaneous fat of the LIN and ALG MEDIUM groups did not differ from the SOY group (P>0.05). Again, DHA proportions in the FISH group and ALG groups were higher compared to the SOY and LIN group (P<0.05). In addition for the ALG groups, increasing DHA proportions with increasing amounts of microalgae in the feed were found. The DHA proportions in the subcutaneous fat of the SOY and LIN groups were respectively 30 and 13 fold lower compared to the ALG HIGH group. Results concerning DPA were not analogous with the fresh meat: while in the meat samples the FISH and LIN group showed the highest and comparable proportions of DPA, in the subcutaneous fat the highest DPA proportion was found in the subcutaneous fat of the FISH group, which was three fold higher than the proportion of the LIN group (P<0.05). The LA proportion was again the highest in the LIN group and the lowest in the ALG LOW group, while the AA proportion did not differ among groups except for almost a two fold higher proportion in the ALG HIGH group compared to all other groups. It should also be noted that the subcutaneous fat of all groups contained lower proportions of long chain n-3 and n-6 PUFA compared to the fresh meat, while around two fold higher proportions of ALA and similar proportions of LA were found in the subcutaneous fat compared to the corresponding fresh meat samples.

Table 5.4. Fatty acid composition of subcutaneous fat (g FA/100 g total FA; n=6)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
C18:3n-3	1.40[b]	2.92[a]	1.28[b]	1.37[b]	1.22[b]	1.24[b]	0.108	<0.001
C20:5n-3	0.022[c]	0.039[c]	0.350[a]	0.041[c]	0.065[c]	0.144[b]	0.020	<0.001
C22:5n-3	0.146[d]	0.209[cd]	0.631[a]	0.176[cd]	0.215[c]	0.372[b]	0.029	<0.001
C22:6n-3	0.034[d]	0.100[d]	0.567[c]	0.409[c]	0.879[b]	1.87[a]	0.106	<0.001
C18:2n-6	14.9[ab]	16.0[a]	13.1[bc]	11.3[c]	13.1[bc]	14.2[ab]	0.354	<0.001
C20:4n-6	0.294[b]	0.274[b]	0.240[b]	0.222[b]	0.294[b]	0.411[a]	0.012	<0.001
PUFA	18.0[abc]	20.8[a]	17.0[bc]	14.6[c]	17.0[bc]	19.8[ab]	0.458	<0.001
SFA	33.2[b]	32.9[b]	34.6[b]	39.3[a]	37.7[a]	38.4[a]	0.508	<0.001
MUFA	45.4[a]	42.4[a]	44.5[a]	43.5[a]	42.4[a]	38.9[b]	0.457	<0.001
Total FA (g/100 g fat)	62.1	59.9	63.9	65.0	63.3	62.1	0.961	0.760

SOY= soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);

SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0;

MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1;

PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3;

SEM= standard error of the mean calculated from all groups (n=36);

[a-d]Values with different letters in the same row indicate significant differences (P<0.05).

Table 5.5 shows the fatty acid composition of the dry fermented sausages. Again, the LIN diet resulted in higher ALA proportions in the dry fermented sausages compared to all other groups and higher EPA proportions were found in the FISH group (both P<0.05). Also the effect of EPA and DHA was still present in the dry fermented sausages: higher concentrations of microalgae in the experimental diets resulted in higher proportions of EPA and DHA in the dry fermented sausages. However, the DHA proportion of the sausages from the ALG LOW group was lower than for the FISH group (P<0.05), whereas the sausages of the ALG HIGH group contained an almost 3 fold higher DHA proportion compared to the FISH group. It is clear that the fatty acid profile of the dry fermented sausages is a result of the fatty acid composition of the fresh meat and the subcutaneous fat.

Table 5.5. Fatty acid composition of dry fermented sausages (g FA/100 g total FA; n=3)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
C18:3n-3	1.36^{cd}	2.73^a	1.31^d	1.53^b	1.40^c	1.12^e	0.124	<0.001
C20:5n-3	0.044^e	0.072^d	0.353^a	0.067^d	0.181^b	0.139^c	0.028	<0.001
C22:5n-3	0.194^c	0.361^b	0.687^a	0.240^c	0.328^b	0.349^b	0.043	<0.001
C22:6n-3	0.047^e	0.106^e	0.579^c	0.412^d	0.911^b	1.385^a	0.123	<0.001
C18:2n-6	14.8^a	14.2^{ab}	13.5^b	11.5^d	14.4^a	12.9^c	0.272	<0.001
C20:4n-6	0.444^a	0.401^b	0.355^c	0.393^b	0.472^a	0.473^a	0.012	<0.001
PUFA	18.1^a	19.1^a	17.8^b	15.3^c	18.9^a	17.8^b	0.295	<0.001
SFA	35.3^b	34.4^b	35.5^b	39.6^a	38.9^a	39.0^a	0.533	<0.001
MUFA	43.0^a	41.6^{ab}	42.8^a	40.6^{abc}	38.4^c	39.9^{bc}	0.473	<0.001
Total FA (g/100g sausage)	26.4	24.4	27.2	27.1	25.7	29.0	0.494	0.123

SOY= soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);

SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0;

MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1;

PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3;

SEM= standard error of the mean calculated from all groups (n=18);

[a-e]Values with different letters in the same row indicate significant differences (P<0.05).

Table 5.6 shows the fatty acid composition of the dry cured hams. The fatty acid composition of the diets was again clearly reflected in the dry cured hams and similar results as for the fresh meat were found. The LIN diet resulted in a higher ALA proportion compared to all other groups ($P<0.05$). The EPA proportion was the highest in the dry cured hams of the FISH group ($P<0.05$), with a seven fold higher proportion compared to the SOY group. Also in the dry cured hams a relatively high proportion of EPA in the ALG HIGH group was found. The EPA proportion of the LIN group was three fold lower compared to the FISH group ($P<0.05$), but 2.5 fold higher compared to the SOY group ($P<0.05$) and did not differ from ALG LOW and ALG MEDIUM. The highest DHA proportion was found in the ALG HIGH group, which was 17 fold higher compared to the SOY group. The DHA proportions of the FISH and ALG MEDIUM group were similar and significantly higher than the SOY and LIN group. The DHA content in the dry cured hams increased with increasing amounts of microalgae in the feeds. For the ALG LOW group, the DHA proportion was three fold lower compared to the ALG HIGH group, but two fold higher compared to the LIN group. The DPA proportions in the dry cured hams was in the order of FISH>LIN>all ALG=SOY. For the n-6 FA, the highest proportions of LA and AA were found in the LIN group. It should be noted that for all groups the proportions of ALA were higher in the dry cured hams compared to the fresh meat, while on the contrary all long chain n-3 PUFA and AA were lower in the dry cured hams.

Table 5.6. Fatty acid composition of trimmed dry cured hams (g FA/100 g total FA; n=6)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
C18:3n-3	0.910[bc]	1.92[a]	1.00[b]	1.01[b]	0.868[bc]	0.776[c]	0.068	<0.001
C20:5n-3	0.125[d]	0.321[c]	0.907[a]	0.202[cd]	0.288[cd]	0.508[b]	0.047	<0.001
C22:5n-3	0.378[c]	0.565[b]	0.858[a]	0.326[c]	0.321[c]	0.407[c]	0.034	<0.001
C22:6n-3	0.105[d]	0.274[d]	0.836[b]	0.614[c]	1.03[b]	1.70[a]	0.090	<0.001
C18:2n-6	13.3[ab]	15.7[a]	13.5[ab]	11.0[b]	11.9[b]	12.5[b]	0.353	<0.001
C20:4n-6	2.35[ab]	2.88[a]	2.01[b]	1.92[b]	2.14[ab]	2.51[ab]	0.090	0.010
PUFA	18.4[b]	23.3[a]	20.3[ab]	16.3[b]	17.9[b]	19.9[ab]	0.526	<0,001
SFA	32.2[b]	30.8[b]	31.6[b]	35.6[a]	35.1[a]	34.5[a]	0.348	<0,001
MUFA	42.7[a]	38.2[b]	40.9[ab]	41.9[ab]	40.6[ab]	38.9[ab]	0.462	0.039
Total FA (g/100 g ham)	4.03	3.05	3.50	3.79	3.62	3.12	0.123	0.161

SOY= soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);

SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0;

MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1;

PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3;

SEM= standard error of the mean calculated from all groups (n=36);

[a-d]Values with different letters in the same row indicate significant differences (P<0.05).

To verify the potential nutritional benefit of the enriched meat and meat products, the amount of EPA+DHA per 100 g meat was calculated. The amount of EPA+DHA per 100 kcal and %RNI was derived from those results (Table 5.7). For the fresh meat, a two to three fold higher intake of EPA+DHA was found for the LIN and ALG LOW group compared to the SOY group. For ALG HIGH and the FISH group even a six fold increase of EPA+DHA was achieved in the fresh meat cuts. Taking into account a recommended daily intake of 667 mg EPA+DHA and an intake portion of 50 g of meat, this results in a %RNI of 2.2% for the fresh meat from the ALG HIGH group instead of 0.3% from the SOY group. However, no claim for an n-3 PUFA enriched meat product could be made.

For the dry fermented sausages all diets resulted in a significantly higher EPA+DHA content compared to the SOY group, varying from a two fold increase for the LIN group to even an 18 fold increase for the ALG HIGH group. The latter gave rise to a %RNI of 34%, which is one third of the daily recommended intake. The dry fermented sausages from the FISH, ALG LOW and ALG MEDIUM groups could be claimed as "source of n-3 PUFA" and from the ALG HIGH group as "food product high in n-3 PUFA".

The dry cured hams originating from pigs fed the FISH and ALG diets, had three to seven fold higher concentrations of EPA+DHA compared to the SOY fed group, resulting in the same increase for %RNI. Still, only the dry cured hams from the FISH and ALG HIGH groups could be claimed "source of n-3 PUFA".

Considering the ALA content of all samples, only the dry fermented sausages contained more than 0.3 g ALA/100 g sausage (data not shown). However, they did not contain more than 0.3 g mg ALA per 100 kcal, so no claim based on the ALA content could be made.

Table 5.7. Nutritional value of the n-3 PUFA enriched meat and meat products

	SOY	LIN	FISH	ALG low	ALG medium	ALG high
Fresh meat						
EPA+DHA (mg/100 g product)	4.46	8.76	26.7	13.1	23.6	29.0
EPA+DHA (mg/100 kcal)[1]	4.02	7.89	24.0	11.8	21.2	26.2
Claim[2]	-	-	-	-	-	-
%RNI[3]	0.3	0.7	2.0	1.0	1.8	2.2
Dry fermented sausage						
EPA+DHA (mg/100 g product)	24.8	45.7	263	135	291	458
EPA+DHA (mg/100 kcal)	6.34	11.7	67.3	34.7	74.6	117
Claim	-	-	source	source	source	high
%RNI	1.9	3.4	20	10	22	34
Dry cured ham						
EPA+DHA (mg/100 g product)	9.93	19. 8	64.7	32.9	50.6	72.8
EPA+DHA (mg/100 kcal)	6.80	13.6	44.4	22.6	34.7	49.9
Claim	-	-	source	-	-	source
%RNI	0.7	1.5	4.9	2.5	3.8	5.5

[1]Calculated using mean fat and protein content as reported in the result section and the Atwater nutrient conversion factors 9 and 4 kcal/g for fat and protein respectively;

[2]Source of n-3 LC PUFA: > 40 mg EPA+DHA per 100 g product and per 100 kcal;

High in n-3 LC PUFA: > 80 mg EPA+DHA per 100 g product and per 100 kcal;

[3]%RNI was calculated with a daily recommended nutrient intake of 667 mg EPA+DHA (=0.3% of the total daily energy intake) and assuming a daily intake portion of 50 g product.

DISCUSSION

The addition of different n-3 PUFA sources to the diets of pigs alters correspondingly the fatty acid composition of the pig muscle and subcutaneous fat and consequently also the composition of the meat products. A higher presence of n-3 PUFA in fresh pork or processed pork products, after supplying n-3 PUFA through the feed was also found by others: in dry cured sausages and subcutaneous fat after supplying linseed (Warnants et al., 1998), in *longissimus* muscle and subcutaneous fat after supplying algae (Marriott et al., 2002), in dry cured shoulder after supplying algae (Sárraga et al., 2007), in *longissimus* muscle after supplying linseed or fish oil (Haak et al., 2008), and in dry cured ham after supplying linseed oil or extruded linseed (Musella et al., 2009). However, it is difficult to quantitatively compare the incorporation of the FA in the tissues with those found in the present study as different n-3 PUFA concentrations, feeding conditions and pig breeds were used in the studies.

As substantial DHA proportions were incorporated in animal tissues from the microalgae fed groups, it can be concluded that dried microalgae in pig feed can be used as DHA source. A clear dose dependent effect was observed, which was also found in other studies in which marine algae were added to pig feed: Marriott et al. (2002) in different raw muscles, Sardi et al. (2006) in *longissimus* muscle and subcutaneous fat and Meadus et al. (2009) in bacon. In the present study, relatively high proportions of EPA were found in the ALG groups. As these proportions are too high to originate solely from the small quantities present in the feed or from conversion of ALA to EPA (considering the results of the SOY and LIN groups), it is likely that part of the EPA originated from DHA by retroconversion. Retroconversion is a minor metabolic pathway, involving one cycle of β-oxidation (Sprecher et al., 1995). Feeding high concentrations of DHA increased the EPA concentration in rat liver (Kaur et al., 2010) and after high DHA intake, EPA concentrations rose in human plasma phospholipids (von Schacky & Weber, 1985), implying

that retroconversion took place. We assume that the addition of 1.8 to 7.1 g/100 g FA DHA in the present study induced some retroconversion.

Regarding DHA, it is worth mentioning that the DHA proportions in the tissues and meat products of the LIN group did not statistically differ from the SOY group, implying that it is not sufficient supplying only ALA, when the objective is to increase also long chain PUFA in ones diet. On the other hand, compared to the SOY group, a significant increase of EPA and DPA was seen in the LIN group, which compares well with other studies supplying a linseed source in pig feed (Santos et al., 2008; Musella et al., 2009; Haak et al., 2009). According to Brenna (2002) only 25% of administrated ALA is available for the production of very long chain PUFA in humans, whereas the largest part is used for energy. The low conversion of ALA to DHA is mostly explained by the rate-limiting enzyme Δ6-desaturase. As described by Blank et al. (2002), the synthesis of EPA and DPA from ALA requires only one pass of the Δ6-desaturase, while the synthesis of DHA from DPA requires a second pass at the Δ6-desaturase after it is elongated to C24:5n-3. Thus C24:5n-3 would be in direct competition with 18 carbon fatty acids for access to the Δ6-desaturase, which may explain the complex kinetics between dietary ALA and tissue DHA.

Compared to the fresh meat lower proportions of long chain PUFA were found in the subcutaneous fat. This is probably due to relatively high amounts of phospholipids in the intramuscular fat of the fresh meat, while subcutaneous fat consists almost exclusively of triacylglycerols. Phospholipids are characterised by a high PUFA content, as they are constituents of cell membranes, mainly represented by long chain fatty acids (Wood et al., 2008). On a tissue weight basis, however, the content of total and individual PUFA is of course higher for the subcutaneous fat than for the fresh meat as a result of the much higher total fatty acid content. This resulted in higher EPA and DHA content in the dry fermented sausages and for the

FISH and ALG groups it is possible to claim the products "source of" or "high in" n-3 PUFA. The fatty acid profiles of the fresh meat and subcutaneous fat are different, but these differences are not reflected in the fatty acid profile of the dry fermented sausage. For the dry fermented sausages lean shoulder meat was used instead of loin, which could explain these differences. In addition, the drying process can also alter the fatty acid profile of the dry fermented sausages as the loss of water mainly affects the fatty acid content of the meat fraction and to a lesser extend that of the subcutaneous fat. As the dietary treatment was still reflected in the dry fermented sausages, it can be concluded that the processes inherent to the production of dry fermented sausages, such as microbiological activity, lipolysis and oxidation (Warnants et al., 1998), did not greatly alter the fatty acid profile of the sausages. Also dry cured hams are subjected to lipolysis during processing, which can promote lipid oxidation, especially in PUFA (Coutron-Gambotti & Gandemer, 1999). Maybe the long chain fatty acids were more oxidized during ripening compared to the less unsaturated ALA, explaining the higher proportions of ALA and the lower proportions of the long chain unsaturated FA in dry cured ham, compared to fresh meat. However, as different muscles are compared here, caution is needed with this hypothesis. Nevertheless, also after a long ripening period the effect of dietary treatment was still present.

In the present study, relatively low concentrations of n-3 PUFA were added to the feed for costs arguments, however still a significant increase in EPA and DHA content was achieved in the meat products compared to the control treatment. For example, when 50 g of fresh meat, dry fermented sausages or dry cured ham originating from the ALG HIGH group is consumed, the intake of EPA+DHA is respectively 14.5, 229 and 36.4 mg of EPA+DHA. This is respectively 2, 34, and 5% of the recommended daily intake of EPA and DHA (Table 5.7), which is resp. 6.5, 18 and 7.3 fold higher compared to the meat products originating from pigs fed the control diet. It should be mentioned that the recommended daily intake of EPA+DHA varies among countries and ranges between 140 to 667 mg/day depending on the authority guidelines (Aranceta &

Pérez-Rodrigo, 2012), which consequently alters the calculated %RNI. As in Belgium a daily intake of 667 mg EPA+DHA (0.3% of our daily energy intake) is recommended by the Superior Health Council of Belgium (2006), the reported %RNI value is lower than when for example 300 mg EPA+DHA would have been used as recommended daily intake.

According to Sioen et al. (2006), the consumption of total meat, poultry and eggs contribute for 10.1% to the daily intake of total n-3 PUFA and for 5.2% and 11.8% to the daily intake of EPA and DHA respectively for Belgian women. Likewise, Howe et al. (2006) emphasized the importance of meat in the daily intake of long chain n-3 PUFA in Australia. They estimated that 43% of the consumed long chain n-3 PUFA originated from meat, poultry and game, because of the much larger consumption of meat and meat products compared with that of fish and seafood. Giving the fact that pork is the major meat source in most industrialized countries, supplementing pig feeds with microalgae could considerably contribute to the achievement of the recommended daily intake of EPA and DHA, without the need of fish oil.

CONCLUSIONS

This study aimed at increasing the long chain n-3 PUFA of pig meat by including linseed oil, fish oil or microalgae in the pig feed. From the results it is clear that the fatty acid composition of the experimental diets was reflected in the meat products. Moreover, microalgae were found to be an excellent source to considerably increase the DHA content of these products. This outcome is of interest in terms of improving the nutritional value of pork, without the use of fish oil. Furthermore, different processing conditions such as fermentation and a long ripening period did not drastically change the fatty acid profile of dry fermented sausages and dry cured hams. Still, PUFA are more prone to oxidation, which could alter the taste and shelf life of these products and additional research is necessary in this respect.

ACKNOWLEDGEMENTS

The EPA and DHA content of loin, dry fermented sausages and dry cured ham is substantially increased when adding microalgae to the pigs feed (CH5).

But how does this affect the sensory quality of these products?

Part I Lowering the ingoing nitrite dose	Part II Increasing the n-3 PUFA content
CH1 With a dog rose extract	CH5 Role of n-3 PUFA source
CH2 With sodium ascorbate	**CH6 Effect on sensory quality**
CH3 With a pre-converted extract	CH7 Role of α-tocopherol in feed
CH4 Nitrite and protein oxidation?	CH8 Effect on health
CH9 General discussion and future prospects	

CHAPTER 6

MICROALGAE INCLUDED IN THE PIG FEED: EFFECT ON OXIDATIVE STABILITY AND SENSORY QUALITY OF N-3 PUFA ENRICHED FRESH MEAT, DRY FERMENTED SAUSAGE AND DRY CURED HAM

ABSTRACT

This experiment was set up to investigate the sensory quality in terms of lipid oxidation, colour, texture and consumers acceptability of n-3 fatty acids enriched fresh meat cuts (*longissimus dorsi*), dry fermented sausages and dry cured hams. Crossbred pigs were given an experimental diet supplemented with 0.6 g/100g linseed oil (LIN), 0.8 g/100g fish oil (FISH) or dried microalgae (ALG). Three ALG groups were considered: ALG LOW, ALG MEDIUM and ALG HIGH with respectively 0.3, 0.6 and 1.2 g dried microalgae per 100 g feed. In the control group soybean oil was added to the diet. The colour and lipid stability of the fresh meat cuts were not affected by the added n-3 fatty acids. In the n-3 fatty acids enriched dry fermented sausages 1.2 to 1.5 fold higher TBARS values compared to the control were found and colour and texture parameters (measured by hardness and chewiness) were altered. Problems of fishy and rancid off-flavours in the ALG HIGH and FISH groups were reported in the dry fermented sausages from the taste panel survey. The dry cured hams from all ALG treatments had 1.2 fold higher TBARS values and inferior texture scores, however these differences were not noticed by the consumer panelists. There were no colour differences between the control dry cured ham and the n-3 fatty acids enriched hams. The volatile compound (E)-2-penten-1-ol, typical from long chain n-3 fatty acid oxidation was 2.7 fold higher in dry cured ham from the ALG HIGH group compared to the control.

INTRODUCTION

The long chain n-3 polyunsaturated fatty acids (n-3 PUFA), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), exert positive effects on human health (reviewed by Narayan et al., 2006). However, intake of these fatty acids is low in Western societies and direct enrichment of meat with these long chain n-3 PUFA by dietary means could help bridging the gap between the recommended and actual intake. In a previous experiment (chapter 5), we succeeded to considerably increase the EPA and DHA content of fresh meat, dry fermented sausage and dry cured ham by supplementing microalgae in the pig feed, which makes microalgae a sustainable alternative for the widely used fish oil. Unfortunately, the increased degree of unsaturation of these fatty acids makes enriched meat products more susceptible to oxidative damage which can negatively affect the meat quality (Wood et al., 2008), so assessment of the sensory quality traits of n-3 FA enriched meat products is needed. Dry cured hams are particularly susceptible to oxidation due to their long ripening time. While a small amount of oxidation products is required to get the typical aroma of dry cured ham, an excessive oxidation leads to off-flavours (Coutron-Gambotti & Gandemer, 1999). As microalgae are supplemented in this study, the off-flavours from EPA and DHA deserve special attention. Although not as long as dry cured hams, also dry fermented sausages are exposed to a ripening period. Moreover, the fermentation process, mincing and high fat content could also influence the oxidation processes of this product. Pork back-fat is traditionally used in the formulation of dry fermented sausages, due to its relevant contribution to the properties of the final product, including its appearance and consistency (Valencia et al., 2006). Yet, changing the fatty acid composition of the muscular and subcutaneous fat changes the lipid melting point and fat firmness (Wood et al., 2008), which subsequently could influence texture and palatability of the meat products.

The aim of present trial was to further investigate the effect of microalgae, added to the diet of pigs, on the oxidative stability and sensory quality of n-3 FA enriched *longissimus* muscle, dry fermented sausage and dry cured ham. Meat products originating from linseed and fish oil fed pigs were included in the experiment for comparison.

MATERIALS AND METHODS

This study was conducted using the samples obtained from a previous trial (chapter 5). Therefore, for the experimental set-up, sampling, carcass measurements and manufacturing of the meat products please consult chapter 5. Briefly, crossbred pigs were given an experimental diet supplemented with 0.6 g/100g linseed oil (LIN), 0.8 g/100g fish oil (FISH) or dried microalgae (ALG). Three ALG groups were considered: ALG LOW, ALG MEDIUM and ALG HIGH with respectively 0.3, 0.6 and 1.2 g dried microalgae per 100 g feed. In the control group soybean oil was added to the diet (SOY).

1. Colour measurements

Colour coordinates (CIE L*a*b* colour system, 1976) were measured with a HunterLab Miniscan Minolta XE plus spectrocolorimeter (light source of D65, standard observer of 10°, 45°/0° geometry, 1 inch. light surface). The L*, a* and b* values are a measure of lightness, redness and yellowness respectively. Measurements on the loin (M. *longissimus dorsi*, n=10) and subcutaneous fat (n=10) were conducted approximately 30h after slaughter. The loin cuts (slices of 2.5 cm) were subsequently exposed to illuminated chilled display (1000 lux, 4°C) and colour parameters were measured after five days. Measurements on the dry fermented sausages (n=4) and the dry cured hams (n=12, *semimembranosus* muscle) were conducted at room temperature immediately after slicing.

2. Lipid oxidation

Malondialdehyde (MDA), a secondary lipid oxidation product, forms a coloured complex with 2-thiobarbituric acid (TBA) which was determined spectrophotometrically at 532 nm. The *longissimus dorsi* (n=5) and trimmed dry cured hams (n=6) were assessed using a distillation method based on Tarladgis et al. (1960). The *longissimus dorsi* samples were firstly exposed to illuminated chilled display (1000 lux, 4°C) for five days. As the dry fermented sausages (n=3) contain nitrate and nitrite, an extraction method with perchloric acid was applied (Ventanas et al., 2006). Results are expressed as mg MDA/ kg meat.

The peroxidisability index (PI) of the feed was calculated from the fatty acid composition of the feed (reported in Chapter 5) according to Kang et al. (2005). PI = (%monoenoic acid*0·025) + (%dienoic acid*1) + (%trienoic acid * 2) + (%tetraenoic acid*4) + (%pentaenoic acid*6)+ (%hexaenoic acid*8), with 0.025, 1, 2, 4, 6 and 8 being the relative oxidation rates of fatty acids containing 1, 2, 3, 4, 5 or 6 double bonds respectively.

3. Volatile compounds

Volatile compounds of the dry cured hams were assessed by SPME-GC/MS (Solid Phase Micro-Extraction – Gas Chromatography/Mass Spectroscopy), based on Ventanas et al. (2006) with some modifications. The volatile compounds were extracted from the headspace using a carboxen-polydimethylsiloxane (CAR/PDMS) fiber (85 µm thickness) (Supelco, Bellefonte, Pennsylvania, USA). One gram of homogenised trimmed ham was put in a 10 ml vial and volatile compounds were extracted in a heating block for 60 min at 37°C. Extracted volatiles were analyzed using a gas chromatograph (Agilent model 6890N) coupled to a mass-selective detector (Agilent model 5973, Agilent Technologies, Diegem, Belgium). Compounds were resolved on a HP-5 column (30 m × 250 µm × 1 µm, 5% phenyl methyl siloxane, Agilent Technologies, Diegem, Belgium), at an inlet temperature of 280°C. Hydrogen flow was 1.1

ml/min and the temperature program was as follows: 40°C for 10 min, increase at 3°C/min to 170°C, increase at 30°C/min to 250°C and hold for 5 min. N-alkanes were run under the same conditions to calculate the Kovats index (KI). Volatiles were identified first by comparing its mass spectra with those available in the Wiley Mass Spectral Database and then by comparison of the KI with those reported in literature. The area of each peak was integrated using ChemStation software and the total peak area was used as an indicator of volatile generated from the samples. Samples were analyzed in triplicate per feeding group and results are provided in arbitrary area units (AAU×10^6).

4. Sensory analysis

For the dry fermented sausages, a consumer test was carried out at the laboratory of animal nutrition and animal product quality (Melle, Belgium). Eight slices (1 mm thickness) of dry fermented sausage from SOY, ALG LOW and ALG HIGH, were randomly presented to an untrained panel of 56 consumers aged between 15 and 80 years. Simultaneously, two other treatments (T1 and T2) from another experiment were included in the sensory analysis. It was not possible to exclude them from the trial as they were offered simultaneously to the panellists. Therefore, statistical analysis was conducted including these two treatments, but they will not be further discussed. The slices were taken from the refrigerator and distributed on plates 30 minutes before the start of each session so that the slices were at room temperature when consumed. The consumer panellists were asked to score the overall taste of the product on a 5-point scale (1 = very bad; 5 = very good). Panel members were also asked to mention off-flavours. Each consumer had two times four slices of sample sausage that were placed on white plastic dishes, identified by random three-digit numbers and served to the consumer panel at room temperature. On each plate one treatment was offered twice. In total each treatment was tasted 92, 85, 91, 87, 89 times for respectively SOY, T1, T2, ALG LOW and ALG HIGH.

Consumers were isolated in individual booths, and distilled water and unsalted crackers were provided to cleanse their palates between tasting the samples.

For the dry cured ham, the assessments were carried out in a sensory laboratory (VG Sensory, Deinze, Belgium) equipped according to ISO 8589-standards. Forty two male and forty eight female adults who regularly eat dry cured ham were recruited to participate. Each panellist received 4 whole slices (0.5 mm thickness). The slices were taken from the refrigerator and distributed on plates 30 minutes before the start of each session so that the slices were at room temperature when consumed. The slices were randomly coded using a three-digit number. A semi-monadic method was used according to Association Francaise de Normalisation (2000): the slices were assessed one after another, removing the previous slice before the following one was served. The judgments were expressed on a 9-point hedonic scale ranging from disliked extremely (score 1) to excellent (score 9). The evaluated traits were: taste, visual perception, colour of the fat border, odour, mouth feel and overall acceptance of perception. In the beginning of the product assessment, after tasting but before the detailed questions, panellists were also asked to write down spontaneous likes and dislikes.

5. Texture measurements

Texture profile analysis was carried out at room temperature with a TA 500 Texture Analyser (Lloyd Instruments Ltd, Bognor Regis, United Kingdom). The head of the texture analyser was equipped with a massive cylinder of 1.2 cm diameter and was programmed to move vertically at a speed of 100 mm/min. The test consisted of two successive compression ramps to a value of 50% of the unloaded specimen height. Slices of 2.0 cm height were subjected to this two-cycle compression test (n=18 for dry cured ham and n=4 for dry fermented sausages). The parameters hardness (N) and chewiness (N×mm) were determined and represent respectively the maximum

force required to compress the sample and the work needed to masticate the sample for swallowing (Bourne, 1982).

6. Statistical analysis

Results were submitted to one-way ANOVA with diet treatment as fixed effect (SPSS 22.0). Mean differences between groups were tested using the Tukey's post hoc test operating at a 5% level of significance. For the dry fermented sausages, differences in overall taste and frequency of detecting off-flavours were investigated using one-way ANOVA considering treatment as fixed effect. For the sensory analysis of the dry cured hams, a balanced, incomplete block design (BIBD) was used. Differences were considered significant when p<0.05.

RESULTS

1. Colour

Colour L*a*b* values of the *longissimus* muscle and subcutaneous fat 30h after slaughter did not differ among feeding groups (p>0.05; data not shown). Likewise, no differences on the colour parameters were noticed in the *longissimus* muscle after five days of illuminated chilled storage (Table 6.1). On the other hand, dietary treatments did significantly affect the colour of dry fermented sausages and dry cured ham (p<0.05, Table 6.1).

For the L* values of the dry fermented sausages, all ALG and FISH groups did not differ (P>0.05) from the control SOY group, whereas the LIN group was darker (P<0.05). The a* values varied from a maximum of 15.1 in the LIN group to a minimum of 13.1 in the ALG MEDIUM group, but none of the n-3 FA enriched dry fermented sausages differed from the control dry fermented sausage (P>0.05). The b* values of the LIN, FISH and ALG HIGH groups

were 1.1 fold higher (P<0.05), while similar values P>0.05) were found for the ALG LOW and

ALG MEDIUM groups in comparison to the SOY group.

For the dry cured hams, no differences among treatments were noticed for the L* values. There

was a significant effect of diet on the a* and b* values, but differences between treatments were

not consistent. Both lower and higher values for the n-3 feeding groups compared to the SOY

group were found.

Table 6.1. Colour parameters of *longissimus dorsi* (n=10), dry fermented sausage (n=4) and dry cured ham (n=6)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
Longissimus dorsi[1]								
L*	55.2	54.0	53.9	54.6	55.9	54.4	0.212	0.498
a*	9.68	9.55	10.2	10.1	9.39	10.3	0.104	0.518
b*	16.4	16.3	16.8	16.8	16.7	17.0	0.063	0.320
Dry fermented sausages								
L*	47.6[ab]	43.9[c]	45.8[bc]	48.1[a]	48.7[a]	49.3[a]	0.417	<0.001
a*	13.9[abc]	15.1[a]	14.5[ab]	14.1[abc]	13.1[c]	13.6[bc]	0.160	<0.001
b*	9.61[c]	10.6[a]	10.4[ab]	9.86[bc]	9.65[c]	10.6[a]	0.102	<0.001
Dry cured ham								
L*	38.4	38.4	38.5	39.0	36.4	37.2	0.290	0.079
a*	15.0	13.8	15.4	13.4	15.2	14.1	0.211	0.018[2]
b*	11.3[ab]	9.80[b]	12.8[a]	10.3[b]	11.4[ab]	10.5[b]	0.230	0.001

SOY=soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);
SEM = standard error of the mean calculated from all groups;
[1]Measurements were carried out after 5 days of illuminated chilled display (1000 lux, 4°C);
[2]Although p<0.05 according to the ANOVA analysis, no differences were reported by the Tukey Post Hoc test.
[a-c]Values with different letters in the same row indicate significant differences (P<0.05).

2. Lipid oxidation

The TBARS values of the *longissimus* muscle, after five days of illuminated chilled display, were relatively low and did not differ (P>0.05) between treatments (Table 6.2). The higher peroxidisability index of the feed is reflected in a lower oxidative stability of the resulting meat products: 1.2 to 1.5 fold higher TBARS values in the n-3 FA enriched dry fermented sausages and dry cured hams compared to the control were found. However, no significant differences for the LIN and FISH groups compared to the SOY group in both the dry cured hams and dry fermented sausages were noticed and also the dry fermented sausages of the ALG LOW group did not differ from the SOY group (P>0.05). ALG HIGH and ALG MEDIUM showed the highest TBARS values in both dry fermented sausage and dry cured ham.

Table 6.2. Peroxidisability index of the feed and mean TBARS values of the *longissimus dorsi* (n=10), dry fermented sausages (n=3) and dry cured hams (n=6), expressed as mg MDA/kg meat

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
Peroxidisability index								
Feed	41.2	41.7	63.0	55.5	72.1	103	-	-
TBARS								
Longissimus dorsi[1]	0.138	0.132	0.149	0.165	0.147	0.169	0.068	0.725
Dry fermented sausage	0.871[b]	1.02[b]	1.04[b]	1.03[b]	1.40[a]	1.54[a]	0.068	<0.001
Dry cured ham	0.321[b]	0.376[ab]	0.439[ab]	0.468[a]	0.495[a]	0.478[a]	0.015	0.002

SOY=soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);
SEM = standard error of the mean calculated from all groups;
PI =peroxidisability index of the feed calculated from the fatty acid composition of Chapter 5 = (%monoenoic acid×0·025) + (%dienoic acid×1)+ (%trienoic acid×2) + (%tetraenoic acid×4) + (%pentaenoic acid×6)+ (%hexaenoic acid×8); Kang et al. (2005);
[1]Measurements were carried out after five days of illuminated chilled display (1000 lux, 4°C).
[a,b] Values with different letters in the same row indicate significant differences (P<0.05).

Expressed in arbitrary area units, no differences (P>0.05) for hexanal, heptanal and nonanal, n-aldehydes frequently used as lipid oxidation markers, were found in the dry cured hams originating from pigs fed different experimental diets, nor for (Z)-1-octen-3-ol and acetic acid (p>0.05). On the other hand, the amount of (E)-2-penten-1-ol was the lowest in the SOY and ALG LOW groups, being significant lower than those found in ALG HIGH samples (P<0.05). Intermediate values were measured for LIN, FISH and ALG MEDIUM samples. The main volatiles, ethanol, 1-pentanol, 1-hexanol, 2-propanone, 2-pentanone, 2-heptanone, ethyl butanoate and 2,2,4,6,6-pentamethylheptane did not show any significant differences among treatments (P>0.05, data not shown). The remaining volatiles were not quantified nor analysed, as focus in the present study was on oxidation products originating from PUFA.

Similar results were found when expressing the results as percentage of area relative to the total peak area.

Table 6.3. Volatile compounds of the dry cured hams (n=3) expressed as arbitrary area units (AAU×10^6) and as percentage of area relative to the total peak area (%)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
AAU×10^6								
Hexanal	764	494	506	483	845	923	68.9	0.224
Heptanal	85.5	90.4	109	88.9	97.5	105	6.15	0.892
Nonanal	7.92	13.7	8.19	8.33	8.73	16.2	1.44	0.456
Acetic acid	30.0	32.4	32.6	32.2	28.5	32.6	7.2	0.998
(E)-2-penten-1-ol	28.8[b]	52.6[ab]	42.9[ab]	34.9[b]	38.6[ab]	78.4[a]	4.9	0.017
(Z)-1-octen-3-ol	20.7	24.2	16.1	17.2	18.3	29.7	1.65	0.124
%								
Hexanal	8.18	4.74	3.92	5.07	7.67	8.96	0.74	0.242
Heptanal	0.857	0.867	0.825	0.936	0.888	1.02	0.06	0.968
Nonanal	0.106	0.131	0.080	0.086	0.080	0.160	0.012	0.356
Acetic acid	2.45	3.34	5.62	3.28	2.56	3.11	0.54	0.634
(E)-2-penten-1-ol	0.281[b]	0.523[ab]	0.334[b]	0.354[b]	0.354[b]	0.752[a]	0.047	0.008
(Z)-1-octen-3-ol	0.202	0.246	0.126	0.173	0.162	0.287	0.018	0.059

SOY=soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);

SEM = standard error of the mean calculated from all groups (n=18).

[a,b] Values with different letters in the same row indicate significant differences ($P<0.05$).

3. Sensory Analysis

Significant differences (P<0.05) for overall taste of the dry fermented sausages were found by the consumer panellists. Mean consumer scores were 3.61±0.81, 3.24±0.94, 2.81±0.99 for respectively SOY, ALG LOW and ALG HIGH. According to the panellists, the taste of the ALG HIGH sausages was unacceptable and different from ALG LOW and ALG SOY (P<0.05), while the taste of the SOY and ALG LOW groups was acceptable and did not differ among each other. A rancid and fishy taste was found in all groups, however, big differences in frequency were found: in 3.3, 6.8 and 24.4% of the consumed samples a rancid or fishy taste was noticed by the consumers in respectively SOY, ALG LOW and ALG HIGH treated dry fermented sausages. According to VG Sensory, a frequency higher than 10% indicates a remarkable off-taste of the product.

No significant differences between the treatments for the sensory acceptability of the dry cured hams were found (P>0.05). Average values for "overall acceptance of perception", "aspect", "colour fat border", "odour", "mouth feel" and "taste" were respectively 6.56±0.23, 6.56±0.14, 6.26±0.16, 6.27±0.13, 6.57±0.20, 6.48±0.20. The overall acceptance of perception was strongly correlated with mouth feel and taste (r²>0.90). No fishy odour and flavour were detected in any sample by the consumer panel.

4. Texture

The hardness and chewiness of the dry fermented sausages and dry cured hams were affected (P<0.05) by the dietary treatments (Table 6.4). Compared to the SOY group, the dry fermented sausages from the FISH and LIN group were clearly softer (P<0.05), while the sausages from the ALG groups did not differ (P>0.05). The chewiness of the ALG LOW group was comparable to the SOY group (P>0.05), while all other groups had lower values (P<0.05).

Both the hardness and chewiness of the dry cured hams were clearly affected by the long chain n-3 fatty acids, as all ALG groups and the FISH group showed lower values compared to the SOY group (P<0.05). On the contrary, the hardness and chewiness of the LIN group were not affected (P>0.05) when comparing to the SOY group.

Table 6.4. Texture parameters, hardness (N) and chewiness (N×mm), of dry fermented sausages (n=4) and dry cured hams (n=18)

	SOY	LIN	FISH	ALG low	ALG medium	ALG high	SEM	p_{diet}
Dry fermented sausage								
Hardness	56.6[ab]	45.1[c]	44.5[c]	57.2[a]	47.8[bc]	47.7[bc]	1.31	<0.001
Chewiness	225[a]	151[b]	143[b]	202[a]	159[b]	153[b]	7.02	<0.001
Dry cured ham								
Hardness	52.6[a]	49.5[ab]	35.5[c]	33.9[c]	35.9[bc]	37.3[bc]	1.54	<0.001
Chewiness	105[a]	94.9[ab]	56.3[c]	43.8[c]	58.7[bc]	64.4[bc]	4.16	<0.001

SOY=soybean oil fed control group; LIN=linseed fed group; FISH=fish oil fed group; ALG= microalgae fed group with different concentrations of microalgae (low, medium, high);
SEM = standard error of the mean calculated from all groups.
[a-c]Values with different letters in the same row indicate significant differences (P<0.05).

DISCUSSION

The aim of this research was to investigate the oxidative stability and sensory quality of n-3 FA enriched fresh meat, dry fermented sausage and dry cured ham, produced from pigs fed microalgae, rich in DHA.

The limited degree of oxidation in the fresh meat cuts (<0.2 mg MDA/kg muscle) is probably due to the low intermuscular fat content of the muscle (approx. 1.2g/100g meat). According to Wood et al. (2008) TBARS values above 0.5 mg MDA/kg muscle are considered critical for fresh meat since they indicate a level of lipid oxidation products which produce a rancid odour and taste which can be detected by consumers. In addition, the colour and colour stability was not affected by the added algae suggesting that it is possible to increase the EPA and DHA content of fresh meat using microalgae in the pig feed, without major sensory consequences. Likewise, Sárraga et al. (2007) did not find any differences in TBARS values in raw ham containing 2.1 g/100 g FA EPA+DHA (which is similar to the ALG MEDIUM group of the present study) compared to pigs fed a conventional diet. Also, no fishy or rancid odour was found in their DHA enriched cooked ham. Also Marriott et al. (2002) did not find differences in off-flavour, juiciness, and overall flavour of the *semimembranosus* muscle containing 0.55 g/100 g FA EPA+DHA originating from microalgae in the pig feed. Lipid oxidation and myoglobin oxidation in meat are coupled and both reactions appear capable of influencing each other (Faustman et al., 2010). During oxidation of oxymyoglobin, both superoxide anion and hydrogen peroxide are produced and further react with iron to produce hydroxyl radicals. The hydroxyl radical has the ability to penetrate into the hydrophobic lipid region and hence facilitates lipid oxidation. On the other hand, lipid oxidation results in a wide range of aldehyde products, which are reported to induce the oxidation of oxymyoglobin. In addition, metmyoglobin formation is generally greater in the presence of unsaturated aldehydes than their saturated counterparts of equivalent carbon chain length (Chaijan et al., 2008). As in the present study the PUFA profile of

the meat products differed considerably between treatments, it was expected that also different amounts of lipid oxidation products among groups were formed, which consequently would influence the colour stability of the meat products. However, in the present study it was found that the colour parameters were not or only little affected by the PUFA content of the meat.

Many volatiles compounds were retrieved from the dry cured hams, being mainly alcohols, ketones, esters and (branched) alkanes, which already have been characterized in dry cured hams (Garcia-Esteban et al., 2004; Ruiz et al., 1999). Oxidation of unsaturated fatty acids in fish is related to the formation of (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal, (E,E)-2,4-heptadienal and 2,4,7-decatrienal and other volatiles formed during oxidation of fish lipids are 1-penten-3-ol, 1-octen-3-ol, 1,5-octadien-3-one, hexanal, heptanal and 2,6-nonadienal (Iglesias & Medina, 2008). In addition, induced oxidation of fish oil particularly rich in EPA and DHA, resulted in the formation (assessed by SPME) of acetic acid, (Z)-3-octen-1-ol, (E)-2-penten-1-ol, (E,E)-2,4-heptadienal, (E,Z)-2,6-nonadienal, 4-octene, (E,Z)-3,6-nonadien-1-ol, and (Z,Z)- 2,5-pentadien-1-ol, propanoic acid, (Z,Z,Z)-4,6,9-nonadecatriene, 2-undecanone, ethyl decanoate, (E)-2-octene, ethyl dodecanoate, (Z)-9-octadecenal, (Z,Z)-2,5-pentadien-1-ol, (E)-2-pentenal, 1-penten-3-ol, 2-(1-pentenyl) furan (Lee et al., 2003). Therefore, especially these volatile compounds were searched for in the present study. Yet, from all above mentioned volatile compounds, only hexanal, heptanal, acetic acid, (E)-2-penten-3-ol and (Z)-1-octen-3-ol were detected. Other studies investigating the effect of EPA and/or DHA-enriched feed on the volatile profile of meat, however, did find quite a lot of these abovementioned volatiles in poultry (Rymer et al., 2010), beef (Elmore et al., 1999) and lamb (Elmore et al., 2005). Maybe the long time curing process affected the occurrence of these volatiles in the dry cured hams of the present study. To the best of our knowledge, this is the first time that the volatiles of dry cured hams enriched with EPA and DHA originating from algae was investigated. Acetic acid is a product of carbohydrate fermentation (Soto et al., 2008), while hexanal, heptanal and (Z)-1-octen-3-ol originate from the

oxidation of n-6 fatty acids (Meynier et al., 1998). The lack of differences between treatments compare well with the fatty acid profile (See Chapter 5), as the n-6 PUFA content of the dry cured hams was similar between treatments. On the other hand, (E)-2-penten-3-ol is formed from oxidation of EPA and DHA (Lee et al., 2003), which explains the higher occurrence of (E)-2-penten-3-ol in the ALG HIGH group. Similarly, Elmore et al. (2005) found much more (E)-2-penten-3-ol in the headspace of grilled *longissimus* muscle from lambs fed on diets supplemented with algae and fish compared to linseed fed lambs.

The consumer panel did not perceive any differences between the dry cured hams, which is in agreement with the overall low TBARS values and the few differences in volatile compounds. On the other hand, the present results are in contrast with other studies where adverse affects of n-3 fatty acids in dry cured hams were noticed by sensory panellists (following feeding of rapeseed, Pastorelli et al., 2003; pure DHA extracted from fish oil, Sárraga et al., 2007; linseed oil, Santos et al., 2008; extruded linseed, Musella et al., 2009). The n-3 fatty acid percentages of the dry cured hams in the present study were similar or even higher compared to the above mentioned studies (except for Santos et al., 2008). Most likely, the difference in total n-3 fatty acid content per 100 g of dry cured ham, due to differences in fat content, explain the differences between the present results and the cited studies for the consumers' acceptability. The fat content of the dry cured hams may greatly differ according to slaughter weight, pig genotype and ripening period of the dry cured hams. Musella et al. (2009) suggested that dietary addition of antioxidants can preserve the long chain fatty acids in products with a long shelf life such as dry cured ham. Possibly the high supplementation of α-tocopheryl acetate in the present study (150 mg/kg) delayed the oxidation processes and consequently influenced positively the sensory characteristics of the dry cured hams. Santos et al. (2008) reported no adverse effects on sensory traits of dry cured hams originating from pigs fed linseed oil with 220 mg/kg α-tocopheryl acetate, while dry cured hams with 20 mg/kg α-tocopheryl acetate were not accepted by trained

experts. Also, the expertise of the sensory panellists (trained versus naive persons) could have affected the results of the sensory analysis.

The mechanical characteristics hardness and chewiness can be explained in terms closely related to actual consumer perception: hardness is the force required to compress a substance between the molar teeth and chewiness is the length of time required to masticate a sample at a constant rate of force application, to reduce it to a consistency suitable for swallowing (Bourne, 1982). The n-3 enriched dry cured hams originating from algae fed pigs showed inferior hardness and chewiness compared to the control group, due to their high EPA and DHA content which affects the fat firmness (Wood et al., 2008). In addition, more lipid oxidation was found in those dry cured hams. So even though not noticed by the consumer panel, the increased n-3 FA did to some extent influence the quality parameters of the n-3 enriched dry cured hams. For the dry fermented sasuges, the lowest values for hardness were unexpectedly found in the LIN and FISH groups, which can not be explained by their fatty acid profile or by their crude composition. Other factors must have affected the texture parameters of these sausages, which however can not be determined using the present data

Regarding the n-3 FA enriched dry fermented sausages through high concentrations of dietary algae, distinct fishy and rancid flavour was observed by a great part of the consumer panellists. Apparently, the supplementation of 150 mg/kg α-tocopheryl acetate was not sufficient to protect against lipid oxidation in these dry fermented - high fat - sausages. As the algae fed groups did not contain any fish oil, no fishy off-flavour was expected in these meat products. Although no other study producing dry fermented sausages from pigs fed microalgae was found to confirm this, it appears that oxidation of EPA and DHA contribute to these fishy off-flavours, independently which source was used. Likewise, Meadus et al. (2009) reported problems of off-odours and off-flavours in cured bacon from pigs fed 0.6 g/100 g microalgae, however they did not mention a fishy taste. It should be further explored whether it is possible to circumvent the

negative effects in these highly n-3 FA enriched meat products by either dietary means or processing practices. On the other hand, the investigated quality traits of the dry fermented sausages originating from pigs fed 0.3 g/100g microalgae, did not differ from the control group. This dry fermented sausage contains 135 mg EPA+DHA/100 g product (see chapter 5) and eating 50 g of this dry fermented sausage would contribute five times more to the recommended daily intake of EPA and DHA compared to a conventional dry fermented sausage.

CONCLUSIONS

Results demonstrate that it is possible to produce fresh meat cuts and long ripened dry cured ham from pigs fed the up to 1.2 g/100 microalgae with improved nutritional properties and minor negative effects on the sensory properties. Dry fermented sausages produced from pigs fed the lowest dose of microalgae (0.3 g/kg) had a similar quality to the conventional dry fermented sausages, but with still a five fold higher concentration of EPA and DHA. Feeding pigs 0.6 and 1.2 g/100 g microalgae resulted in unacceptable dry fermented sausages.

ACKNOWLEDGEMENTS

Dry fermented sausages enriched with n-3 PUFA have inferior sensory quality compared to conventional sausages (CH6).

Does supplementation of supra-nutritional α-tocopherol doses in the feed improve the oxidative stability?

Part I Lowering the ingoing nitrite dose	Part II Increasing the n-3 PUFA content
CH1 With a dog rose extract	CH5 Role of n-3 PUFA source
CH2 With sodium ascorbate	CH6 Effect on sensory quality
CH3 With a pre-converted extract	CH7 Role of α-tocopherol in feed
CH4 Nitrite and protein oxidation?	CH8 Effect on health
	CH9 General discussion and future prospects

OXIDATIVE STABILITY OF PORK, SUBCUTANEOUS FAT AND DRY FERMENTED SAUSAGE FROM PIGS FED DIETS SUPPLEMENTED WITH FISH OIL AND DIFFERENT LEVELS OF ALPHA-TOCOPHERYL ACETATE

ABSTRACT

The effect of feeding supra-nutritional levels of α-tocopheryl acetate on lipid oxidation, colour and colour stability of loin (M. *longissimus dorsi)* and dry fermented sausages enriched with very long chain n-3 polyunsaturated fatty acids was studied. Pigs were fed a diet supplemented with 1.75 g/100 g fish oil and three levels of α-tocopherol: 95, 175 and 400 mg/kg. Loin and subcutaneous α-tocopherol levels were elevated as a result of the dietary α-tocopherol supplementation ($P<0.05$). About two fold higher α-tocopherol concentrations were present in the subcutaneous fat as compared to the corresponding loin samples. During 8 days of chilled display the α-tocopherol concentrations of the loins decreased considerably. Lipid oxidation, as measured by thiobarbituric acid reactive substances, in the loin and dry fermented sausages was not improved ($P>0.05$). However, in the subcutaneous fat reduced lipid oxidation was found when comparing 95 mg/kg versus 175 and 400 mg/kg α-tocopherol ($P<0.05$) supplementation after induced oxidation (seven days at 30°C). Initial colour L*, a*, b* values of the loin and subcutaneous fat were not affected ($P>0.05$), while some modest differences ($P<0.05$) in the dry fermented sausages indicate a slight positive effect of the α-tocopherol supplementation. Colour stability of defrosted loins and dry fermented sausages was not affected by the investigated dietary α-tocopherol levels ($P>0.05$).

INTRODUCTION

The nutritional recommendation for optimum animal performance under normal management and environmental conditions in fattening pigs stipulates the use of 15-40 mg all-rac-α-tocopheryl acetate/kg feed. However, numerous studies have shown that the use of supra-nutritional levels of α-tocopherol is an efficient approach to improve the quality and storage stability of pork (reviewed by Jensen et al., 1998). Alpha-tocopherol is considered as the primary lipid soluble antioxidant in biological systems, which acts by disrupting the chain of lipid oxidation in cell membranes, thus preventing the formation of lipid hydroperoxides (Bramley et al., 2000). The presence of α-tocopherol in membranes, where lipid oxidation is initiated, allows it to function very efficiently compared to other antioxidants (Lauridsen et al., 1997). Dietary supplementation results in the incorporation of α-tocopherol within membranes, making this a more effective approach than adding α-tocopherol *post-mortem* to meat. The *post-mortem* exogenous addition probably results in extensive surface contact with muscle, but this contact is only superficial and not intramembranal; only prolonged dietary supplementation allows for incorporation of α-tocopherol into subcellular compartments (Liu et al., 1995). The effect of dietary α-tocopherol on lipid and colour stability in fresh pork has been extensively studied and has been shown to be effective (Jensen et al., 1998; Sales & Koukolova, 2011; Trefan et al., 2011).

The increasing awareness of the need to augment the daily intake of n-3 polyunsaturated fatty acids (n-3 PUFA) and especially the very long chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for health reasons (Simopoulos, 1999), has prompted research into means of manipulating the fatty acid composition of meat. As these n-3 PUFA are more prone to oxidation (Wood et al., 2008), it can be hypothesized that increasing n-3 fatty acid levels requires increased levels of dietary antioxidants, such as α-tocopherol, to avoid negative effects on meat quality. Positive effects of supra-nutritional supplementation of α-tocopherol on the

oxidative stability of n-3 PUFA enriched fresh pork have been reported (Onibi et al., 2000; Rey et al., 2001; Juárez et al., 2011; Botsoglou et al., 2012; Sobotka et al., 2012; Botsoglou et al., 2014), but its effect on the oxidative stability of n-3 PUFA enriched meat products is less well documented (Hoz et al., 2004). As the antioxidant effect of endogenous α-tocopherol in meat products may depend on their fat content, it should be investigated if supplementation of α-tocopherol also enhances the oxidative stability of n-3 PUFA enriched meat products high in fat. The objective of this study was to investigate the colour and lipid oxidative stability of dry fermented sausages produced from pigs fed fish oil and high levels of α-tocopherol.

MATERIALS AND METHODS

This experiment was conducted simultaneously with the experiment of chapter 5. The experimental set-up, sampling, carcass measurements and manufacturing of the meat products and chemical analyses are therefore generally the same. However, the material and methods section is still included because of some experimental differences.

1. Experimental set-up and sampling

Three groups of ten female crossbred pigs each were fattened under commercial conditions on different diets from 75 kg until 110 kg live weight. Water and feed was offered *ad libitum*. The feed was based on barley, wheat and soybean meal and manufactured by Lambers-Seghers (Baasrode, Belgium). The three experimental diets consisted of different levels of α-tocopheryl acetate (50, 150, 300 mg/kg feed) and 1.75 % fish oil (g/100 g feed) to increase the very long chain n-3 PUFA content of the pork products. All diets were formulated to an equal fat content (5.1%, as fed) and energy supply (2270 kcal/kg, as fed) and supplemented with 0.4 mg/kg organic selenium (Selplex, Alltech, Deinze, Belgium). The fish oil was supplied by INVE Technologies NV (Dendermonde, Belgium) and other ingredients were purchased on the local

market. The composition of the experimental diets is presented in Table 7.1. Feed samples were collected for fatty acid analysis and α-tocopherol content. As the α-tocopherol analysis revealed that higher amounts of α-tocopheryl acetate were supplemented instead of the proposed 50, 150 and 300 mg/kg, treatments are referred to as T95, T175 and T400 with respectively 95, 175 and 400 mg/kg α-tocopherol in the feed.

All pigs (ten per treatment) were slaughtered at approximately 110 kg live weight in a commercial slaughterhouse after electrical stunning (Westvlees, Westrozebeke, Belgium). After slaughtering and cooling for 24h, loin samples were obtained from M. *longissimus dorsi* and subcutaneous fat was taken from the back-fat. The samples were transported to the laboratory and part of them were used for colour measurements (approximately 30h after slaughter). The other samples were stored vacuum packed at -20°C until analysis. From three animals per dietary group also left shoulder meat and additional subcutaneous fat was sampled and pooled for the production of dry fermented sausages.

Table 7.1. Composition of the feed (g/100g feed, % as fed)

	T95	T175	T400
Corn	15.0	15.0	15.0
Wheat gluten	14.7	14.5	14.2
Barley	15.0	15.0	15.0
Wheat	15.0	15.0	15.0
Wheat shorts	12.0	12.0	12.0
Sorghum	11.0	11.0	11.0
Soybean scrap 47% CP	8.7	8.7	8.7
Peas	3.0	3.0	3.0
Rendered Animal Fat	0.28	0.28	0.28
L-Threonine	0.09	0.09	0.09
DL-Methionine	0.02	0.02	0.02
L-Lysine	1.0	1.0	1.0
Ca carbonate 38%	1.15	1.15	1.15
Vitamin-mineral premix[1]	1.00	1.00	1.00
α-tocopheryl acetate 5%[2]	0.00	0.20	0.50
Soy oil	0.27	0.27	0.27
Fish oil	1.75	1.75	1.75
Energy (kcal, as fed)	2270	2270	2270

T95, T175, T400: experimental feed supplemented with fish oil and respectively 50, 150 and 300 α-tocopheryl acetate (mg/kg feed).
[1]Vitamin-mineral premix ("kern" Lambers-Segehrs of Nutreco) contained 5 g/kg α-tocopheryl acetate and 0.04 g/kg organic selenium (Sellplex).
[2]α-tocopheryl acetate concentrate contained 50 g/kg α-tocopheryl acetate.

2. Carcass measurements

Warm carcass weight and carcass lean meat percentage was assessed at the slaughterhouse for all pigs (n=30). At 24h *post mortem*, pH (Knick portamess 654, Knick Elektronische Messgeräte GmbH, Berlin, Germany) with Schott N5800A electrode (Schott Instruments, Mainz, Germany) and conductivity (Pork Quality Meter, Kombi, Intek GmbH, Aichach, Germany) were measured in the loin (M. *longissimus dorsi*).

3. Production of dry fermented sausage

Dry fermented sausages were produced at Ter Groene Poorte (Brugge, Belgium) using a standard commercial recipe. For each treatment, a batch was made that consisted of 2.1 kg lean shoulder meat, 0.9 kg subcutaneous fat, 84 g NaCl, 3 g KNO_3, 30 g spices (Raps, Beringen, Belgium) and 1.5 g starter culture (Biosprint from Raps, Beringen Belgium). All sausages were prepared on the same day. Frozen shoulder meat from three different animals was minced in a bowl chopper, together with all additives, except NaCl. Subsequently, the frozen subcutaneous fat was added and mincing was continued until a homogenous batter was obtained. Then, NaCl was included and the batter was further chopped until a temperature of -2°C was reached. The mixture was stuffed into 6 cm diameter casings, hand-linked at 30 cm and the sausages were left for six hours at ambient temperature (day 1). After that, the sausages were placed in a fermentation chamber and the following conditions of temperature and relative humidity (RH) were applied: 24°C and 95% RH (day 2), 23°C and 92% RH (day 3), 21°C and 90% RH and smoking for one hour (day 4), 20°C and 88% RH (day 5 until day 7) and from day 8 until day 23 the temperature was kept at 17°C with 76% RH. Finally, the dry fermented sausages were vacuum packed and stored at 4°C for colour measurements and at -20°C for chemical analyses.

4. Proximate composition

Dry matter, crude protein and crude fat content of the fresh meat and dry fermented sausage (n=2) were determined according to the ISO 1442-1973, ISO 937-1978 and ISO 1444-1973 methods, respectively. Fresh meat samples were taken at random from three animals and analyses were carried out in single (n=3), while one dry fermented sausage, chosen at random, was analysed in duplicate (n=2). Results were expressed as g/100 g fresh matter. For the fatty acid analysis, lipids were extracted from the samples using chloroform/methanol (2/1; v/v) (modified after Folch et al., 1957). Fatty acids were methylated according to Raes et al. (2001) and analysed by gas chromatography (HP6890, Brussels, Belgium) on a CP-Sil88 column (100 m × 0.25 mm × 0.25 µm; Chrompack, The Netherlands). Peaks were identified based on their retention times, corresponding with standards (NuChek Prep., IL, USA; Sigma, Bornem, Belgium). Nonadecanoic acid (C19:0) was used as an internal standard to quantify the individual and total fatty acids. A number of samples were taken at random per treatment, i.e. n=5 for fresh meat and n=3 for dry fermented sausage, while pooled samples of six animals (equal weight) were analysed in duplicate for the subcutaneous fat (n=2). The fatty acid profiles were expressed in g/100 g of total fatty acid methyl esters and total fatty acid content is expressed as g FA/100 g fresh matter.

The α-tocopherol content of the loins was determined according to the method of Desai (1984) with slight modifications and involves a saponification step and hexane extraction. Since saponification often leads to losses of α-tocopherol in feed and samples with a high fat content, a method for the determination of α-tocopherol in feed and subcutaneous fat was developed without a saponification step (Claeys et al., 2014). In this method, α-tocopherol was extracted with hot ethanol, and the co-extracted fat was removed by centrifugation. Removal of the fat fraction was made possible by the addition of water, to achieve an ethanol/water ratio of 40/7,

followed by cooling on ice before centrifugation. The α-tocopherol was extracted from the ethanol fraction with hexane.

For all matrices the hexane was evaporated using nitrogen gas and the residue was mixed in 1 ml of methanol. All samples were analysed by reversed phase HPLC (GE Healthcare, Diegem, Belgium), using a Supelcosil LC18 column (25 cm × 4.6 mm × 5 µm; Sigma-Aldrich, Bornem, Belgium). The mobile phase was a mixture of methanol/water (97/3; v/v) and the elution was performed at a flow rate of 2.0 ml/min. UV-detection was accomplished at a wavelength of 292 nm. The α-tocopherol content of the samples was determined by comparison of peak areas with those obtained from a standard curve of α-tocopherol. The results were expressed as mg α-tocopherol/kg sample.

5. Oxidative stability

Lipid oxidation was assessed by the TBARS method. Malondialdehyde, a secondary lipid oxidation product, forms a coloured complex with 2-thiobarbituric acid (TBA) which was determined spectrophotometrically at 532 nm. The loin and subcutaneous fat samples were assessed using a distillation method based on Tarladgis et al. (1960). Before the distillation step, the meat or fat was homogenized with water and butylhydroxytoluene was added as antioxidant. The fresh loin samples (n=5) were never frozen, but exposed approximately 30h after slaughter to illuminated chilled display (1000 lux, 4°C) for five days. Slices of 2.5 cm were wrapped in an oxygen permeable plastic foil. Other loin slices (n=6) were frozen at -18°C for nine months and after thawing subjected to the same display conditions (1000 lux, 4°C) for eight days. To induce lipid oxidation in the subcutaneous fat, five grams were weighted in a plastic jar, covered with an oxygen permeable plastic foil and left in an oven at 30°C in dark for seven days. The dry fermented sausages (n=3) were immediately frozen after ripening and as they contain nitrate and

nitrite, an extraction method with perchloric acid was applied (Ventanas et al., 2006). Results were expressed as mg malondialdehyde/ kg meat.

6. Colour stability

Colour coordinates (CIE L*a*b*colour system, 1976) were measured with a HunterLab Miniscan Minolta XE plus spectrocolorimeter (light source of D65, standard observer of 10°, 45°/0° geometry, 1 inch. light surface). The L*, a* and b* values (CIE L*a*b* colour system) were assessed as a measure of respectively lightness, redness and yellowness. Measurements on the loin (n=10) and subcutaneous fat (n=10) were conducted approximately 30h after slaughter. For colour stability measurements, defrosted loin cuts (slices of 2.5 cm) were wrapped in an oxygen permeable foil and subjected to illuminated chilled display (1000 lux, 4°C) during eight days. Measurements on the dry fermented sausages (n=4) were conducted at room temperature immediately after slicing and each day for the following seven days. Before slicing, the whole dry fermented sausages were allowed to achieve room temperature and before the first colour measurements, slices were wrapped in an oxygen permeable plastic foil.

The colour fading of the defrosted loins was measured as a decline in a* values from day 0 until day 8 and data were fitted to a non-linear, one-phase exponential decay curve. In general, exponential decay equation models many chemical and biological processes. It is used whenever the rate at which something happens is proportional to the amount which is left. The equation for an one-phase exponential decay curve is:

$Y = (Y_0 - \text{plateau}) \times \exp(-K \times X) + \text{Plateau}$

with Y_0 = initial a* value (intercept)

        Plateau = the ultimate a* value

        K (expressed in inverse minutes) = the rate constant

As the dry fermented sausages have a high fat content and due to oxidation this fat becomes yellowish during display, it was more suitable to report the increased yellowness of the dry fermented sausage for colour stability. The b* values from day 0 until day 7 were fitted to a non-linear, one-phase exponential association curve (GraphPad Prism6, Demo 2014). However, generally this equation describes the pseudo-first order association kinetics of the interaction between a ligand and its receptor. During each time interval a certain fraction of the unoccupied receptors become occupied. But as time advances, fewer receptors are unoccupied so fewer ligand bind and the curve levels off. The equation for an one-phase exponential association curve is:

$$Y = Y_0 + (\text{plateau} - Y_0) \times (1 - \exp(-K \times X))$$

with Y_0 = initial b* value (intercept)

Plateau = the ultimate b* value

K (expressed in inverse minutes) = the rate constant

The measurements were fitted separately per replicate, and the fitted parameters for Y_0, plateau, and K were further used for statistical analyses. The goodness of fit was checked by the R^2 value, which was at least 0.90 for the defrosted loin and 0.98 for the dry fermented sausages. Four ambiguous fittings were found for the defrosted loins, which were not included in the statistical analyses.

7. Statistical analysis

The data were submitted to analysis of variance with diet as main effect (SPSS Statistics 22.0). A new factor combining exposure day with diet was made to assess the differences between the α-tocopherol content of the unexposed and the exposed loin samples and one-way ANOVA was

used. Mean differences between groups were tested using the Tukey post hoc test operating at a 5% level of significance.

RESULTS

Relatively high concentrations of EPA and DHA, originating from the supplemented fish oil, were found in the diets (Table 7.2). We did not succeed at adding 50, 150 and 300 mg/kg α-tocopherol in the feed, instead 93, 175 and 400 mg/kg α-tocopherol was recovered (Table 6.2). This implies that there is no control treatment with a basal level of 50 mg/kg α-tocopherol. Luckily, still clearly different levels of dietary α-tocopherol were supplied.

Table 7.2. Fatty acid composition (g FA/100 g total FA) and α-tocopherol content (mg/kg) of the experimental diets

	T95	T175	T400
C18:3n-3	3.16	3.15	3.07
C20:5n-3	5.78	6.70	6.58
C22:5n-3	0.73	0.87	0.83
C22:6n-3	5.03	5.93	5.66
C18:2n-6	31.9	30.2	29.6
C20:4n-6	<0.10	<0.10	<0.10
PUFA	48.0	48.5	47.4
SFA	23.3	22.9	24.0
MUFA	23.1	22.8	22.9
Total FA (g/100 g feed)	4.07	4.28	4.51
α-tocopherol	93.6	175	400

T95, T175, T400: experimental feed supplemented with fish oil and respectively 95, 175 and 400 mg/kg α-tocopherol; PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3;
SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0;
MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1.

The mean (± standard deviation) warm carcass weight (102 ± 6 kg) and carcass lean meat percentage (62.0 ± 2.7%) did not differ between feeding groups (P>0.05). Likewise, conductivity in the loin (4.59 ± 0.95) was not influenced by the diet (P>0.05) and nor was the pH value (5.57 ± 0.09). For the loin, no effect of diet was found for dry matter (29.2 ± 0.9 g/100 g meat), crude fat (1.07 ± 0.46 g/100 g meat) and crude protein (25.0 ± 0.9 g/100 g meat) content (P>0.05). Similarly, no differences were found among groups for the dry matter (62.6 ± 1.5), crude fat (30.4 ± 1.8) and crude protein (23.9 ± 1.2) content of the dry fermented sausages (P>0.05).

Relatively high amounts of EPA, DPA and DHA were found in the loin, subcutaneous fat and dry fermented sausages (Table 7.3). No effect of α-tocopherol supplementation on the FA profile of the loin was observed (P>0.05), while a significant effect was found in the subcutaneous fat and in the dry fermented sausages (P<0.05). However, the differences between treatments were limited in magnitude and are not further discussed.

Table 7.3. Fatty acid profile (g/100 g total fatty acids) of the loin (M. *longissimus dorsi,* n=5), subcutaneous fat (pooled sample, n=2) and dry fermented sausage (n=3)

	T95	T175	T400	SEM	p_{diet}
Loin					
C18:3n-3	0.522	0.500	0.548	0.021	0.689
C20:5n-3	2.12	1.93	2.23	0.179	0.820
C22:5n-3	1.01	1.05	1.04	0.067	0.970
C22:6n-3	1.31	1.39	1.34	0.104	0.955
C18:2n-6	12.0	11.1	12.0	0.756	0.861
C20:4n-6	2.36	2.28	2.32	0.231	0.992
PUFA	20.3	19.2	20.5	1.37	0.925
SFA	34.5	34.0	33.8	0.717	0.932
MUFA	41.3	41.8	39.3	1.18	0.690
Total FA (g/100 g loin)	1.33	1.52	1.19	0.093	0.370
Subcutaneous fat					
C18:3n-3	1.29[a]	1.35[ab]	1.37[b]	0.015	0.024
C20:5n-3	0.742[c]	0.833[a]	0.792[b]	0.017	<0.001
C22:5n-3	0.972[ab]	1.02[a]	0.936[b]	0.016	0.020
C22:6n-3	1.13[b]	1.23[a]	1.13[b]	0.021	0.015
C18:2n-6	14.3[b]	14.6[b]	15.0[a]	0.132	0.010
C20:4n-6	0.241	0.243	0.237	0.016	0.306
PUFA	19.8[b]	20.4[a]	20.6[a]	0.149	0.010
SFA	32.9[b]	34.0[a]	33.5[ab]	0.217	0.015
MUFA	41.9[a]	40.5[b]	40.7[b]	0.266	0.006
Total FA (g/100 g fat)	78.1	83.9	88.8	2.27	0.128
Dry fermented sausage					
C18:3n-3	1.30	1.29	1.36	0.013	0.051
C20:5n-3	0.849[b]	0.900[a]	0.907[a]	0.011	0.017
C22:5n-3	1.03[b]	1.13[a]	1.18[a]	0.024	0.004
C22:6n-3	1.15[c]	1.23[b]	1.31[a]	0.022	<0.001
C18:2n-6	14.0[b]	14.1[b]	15.0[a]	0.175	0.032
C20:4n-6	0.375[b]	0.410[a]	0.356[b]	0.009	0.003
PUFA	19.7[b]	20.1[b]	21.1[a]	0.234	0.013
SFA	35.6	36.3	35.1	0.316	0.325
MUFA	39.7	38.5	37.0	0.507	0.089
Total FA (g/100 g sausage)	27.1[a]	23.5[b]	25.7[ab]	0.677	0.019

T95, T175, T400: loin, subcuteneous fat and dry fermented sausages produced from pigs fed fish oil and respectively 95, 175 and 400 mg/kg α-tocopherol;

PUFA = C18:2n-6 + C18:3n-6 + C20:2n-6 + C20:3n-6 + C20:4n-6 + C22:4n-6 + C22:5n-6 + C18:3n-3 + C18:4n-3 + C20:3n-3 + C20:5n-3 +C22:5n-3 + C22:6n-3;

SFA = C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0;

MUFA = C14:1 + C16:1 + C17:1 + C18:1 + C20:1;

SEM = standard error of the mean calculated from all groups;

[a-c]Values with different letters in the same row indicate significant differences (P<0.05).

The α-tocopherol content and lipid oxidation results are summarized in Table 7.4. Increased dietary supplementation of α-tocopheryl acetate resulted in elevated levels of α-tocopherol in the loin and subcutaneous fat (P<0.05). About two fold higher α-tocopherol concentrations were present in the subcutaneous fat compared to the corresponding loin samples. During the eight days of chilled display the α-tocopherol concentrations decreased significantly.

The TBARS values found in defrosted loin cuts after eight days of chilled display were about ten fold higher compared to those found in fresh loin samples after five days of chilled display. For both cases, no effect of the α-tocopherol dose was observed, as no significant differences between treatments were found (P>0.05). On the contrary, TBARS values in the subcutaneous fat from pigs fed 95 mg/kg α-tocopherol were on average two fold higher compared to T175 and T400 samples after seven days of induced oxidation at 30°C. The α-tocopherol level did not influence lipid oxidation in dry fermented sausages (P>0.05).

Table 7.4. α-tocopherol content (mg/kg) and lipid oxidation (mg MDA/kg) in loin (*longissimus dorsi,* n=6), subcutaneous fat (n=6) and dry fermented sausage (n=3)

	T95	T175	T400	SEM	p_{diet}
α-tocopherol content					
Loin	2.43[b,x]	3.13[a,x]	3.31[a,x]	0.117	<0.001
Loin after eight days of display	1.30[b,y]	2.04[a,y]	1.89[ab,y]	0.132	0.036
Subcutaneous fat[1]	4.36[b]	5.23[ab]	7.10[a]	0.457	0.031
Lipid oxidation					
Loin after 5 days of display	0.160	0.164	0.136	0.011	0.557
Defrosted loin after 8 days of display	1.63	1.59	1.76	0.103	0.810
Subcutaneous fat[1]	13.9[a]	5.95[b]	7.96[b]	0.903	0.001
Dry fermented sausage	2.43	1.83	2.32	0.156	0.243

T95, T175, T400: loin, subcuteneous fat and dry fermented sausage produced from pigs fed fish oil and respectively 95, 175 and 400 mg/kg α-tocopherol;
[1]Measurements were carried out after seven days of induced lipid oxidation at 30°C in dark;
SEM = standard error of the mean calculated from all groups;
[a,b]Values with different letters in the same row indicate significant differences (P<0.05);
[x,y]Effect of display on α-tocopherol content of the loins: values with different letters within the same treatment indicate significant differences (P<0.05).

Colour measurements of the unexposed fresh loin and subcutaneous fat did not reveal significant differences among the groups for the L*, a* and b* values (Table 7.5). The dietary α-tocopherol supplementation did not affect the Y0, plateau and rate contant K, obtained from fitting the individual a* values of the defrosted loins to a one-phase decay exponential curve (Figure 6.1). For the dry fermented sausages (Table 7.5 and Figure 7.2), significantly higher L* and lower a* values were found in the sausages produced from pigs fed 400 mg/kg α-tocopherol as compared to those from pigs fed 95 or 175 mg/kg α-tocopherol acetate ($P<0.05$). The b* values at day 0 and the Y0 values obtained from fitting the b* values to a one-phase association exponential curve were different between all three groups ($P<0.05$) and the order was T175<T400<T95, while no effect of dietary α-tocopherol supplementation was observed for the plateau and the rate constant K.

Table 7.5. Colour measures of freshly cut loin (*longissimus dorsi,* n=10), subcutaneous fat (n=) and dry fermented sausage (n=3)

	T95	T175	T400	SEM	p$_{diet}$
L*					
Fresh loin	51.4	51.3	52.7	0.426	0.345
Subcutaneous fat	78.5	78.6	78.2	0.442	0.946
Dry fermented sausage	45.9[b]	46.3[b]	47.8[a]	0.266	0.001
a*					
Fresh loin	7.17	7.27	7.26	0.153	0.962
Subcutaneous fat	3.89	4.11	4.27	0.226	0.792
Dry fermented sausage	14.5[a]	14.3[a]	13.4[b]	0.188	0.010
b*					
Fresh loin	14.3	14.6	14.7	0.124	0.459
Subcutaneous fat	11.7	11.6	12.1	0.184	0.551
Dry fermented sausage	10.5[a]	9.62[c]	9.91[b]	0.115	<0.001

T95, T175, T400: loin, subcuteneous fat and dry fermented sausage produced from pigs fed fish oil and respectively 95, 175 and 400 mg/kg α-tocopherol;
SEM = standard error of the mean calculated from all groups;
[a-c]Values with different letters in the same row indicate significant differences ($P<0.05$).

Defrosted loin
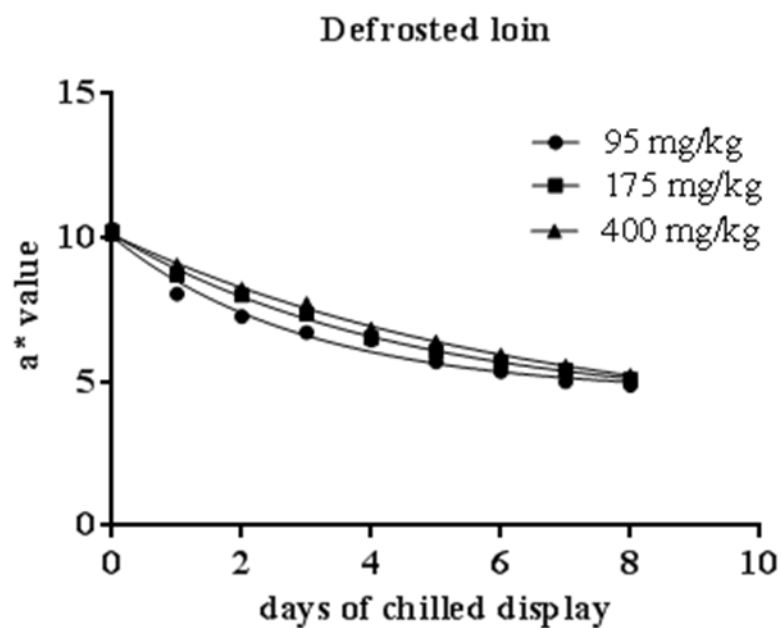
	T95	T175	T400	SEM	p_{diet}
Y0	10.1	10.2	10.9	0.333	0.397
Plateau	4.43	4.16	4.77	0.364	0.849
K	0.415	0.268	0.240	0.051	0.335

Figure 7.1. One-phase exponential decay curve fitted to the mean colour a* values per treatment for illustration purposes. Parameters obtained from the individually fitted data are summarized in the table beneath the figure. T95, T175, T400: defrosted loin cuts produced from pigs fed respectively 95, 175 and 400 mg/kg α-tocopherol. SEM = standard error of the mean calculated from all groups.

Dry fermented sausage
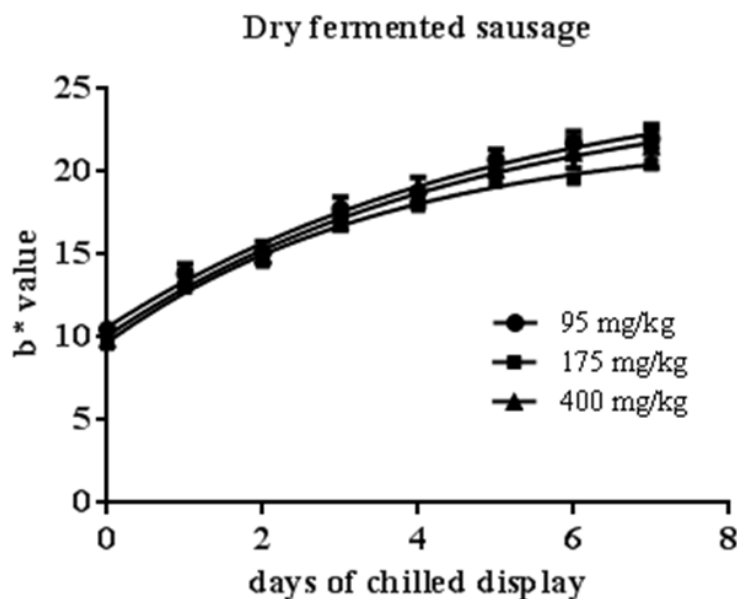
	T95	T175	T400	SEM	p_{diet}
Y0	10.6[a]	9.69[c]	10.1[b]	0.123	0.001
Plateau	27.7	22.5	25.9	1.08	0.132
K	0.196	0.272	0.210	0.019	0.261

Figure 7.2. One-phase exponential association curve was fitted to the mean colour b* values per treatment for illustration purposes. Parameters obtained from the individually fitted data are summarized in the table beneath. T95, T175, T400: dry fermented sausage produced from pigs fed fish oil and respectively 95, 175 and 400 mg/kg α-tocopherol. SEM = standard error of the mean calculated from all groups. [a-c]Values with different letters in the same row indicate significant differences (P<0.05).

DISCUSSION

The purpose of this study was to investigate whether high levels of dietary α-tocopherol can enhance the colour and lipid oxidative stability of n-3 enriched PUFA meat products.

Increased dietary supplementation of α-tocopherol resulted in elevated levels in the M. *Longissimus dorsi* and the results compare well with those reported by Sales & Koukolova (2011) and Trefan et al. (2011). The level of α-tocopherol after dietary intake varies between tissues, which probably depends on the metabolic activities and fat content of the tissue. The highest levels of α-tocopherol are found in the kidney fat and subcutaneous fat, followed by the liver, lung, heart, kidney, muscle and brain (Morrissey, et al., 1996), which explains the higher α-tocopherol content in the subcutaneous fat compared to the loins.

It was expected that the elevated α-tocopherol concentrations in the loin would be reflected in an improved oxidative stability of the loin, but this was not the case. As the α-tocopherol content decreased during illuminated chilled display, which implies that the α-tocopherol was utilized during oxidation processes, it may be suggested that there was no additional benefit of supplementing 175 or 400 mg/kg α-tocopherol compared to the antioxidant effect of 95 mg/kg dietary α-tocopherol. Botsoglou et al. (2014) conducted an analogous experiment, investigating the effect of dietary α-tocopherol (20 versus 200 mg/kg) on lipid oxidation in M. *longissimus dorsi* from pigs fed 2 g/100 g fish oil. Compared to the present study, very similar EPA and DHA proportions (3.6 g/100 g fatty acids) and TBARS values after six days of illuminated chilled display (approx. 0.15 µg MDA/kg meat) were found in the samples supplemented with 200 mg/kg α-tocopheryl acetate, while in the loins of pigs fed only 20 mg/kg α-tocopheryl acetate four fold higher TBARS were found. Extrapolation of these results would imply that in the present study supplementation of 95 mg/kg α-tocopherol did exhibit an antioxidant effect. Regrettably, no control treatment with basal levels of α-tocopherol was included in the present study to verify the antioxidant effect of 95 mg/kg dietary α-tocopherol.

Studies reporting positive effects of dietary α-tocopherol on lipid oxidation in n-3 PUFA enriched pork all compared against a basal diet containing 0-40 mg/kg dietary α-tocopherol (Onibi et al., 2000; Rey et al., 2001; Juárez et al., 2011; Botsoglou et al., 2012; Sobotka et al., 2012; Botsoglou et al., 2014) and no additional benefit of increasing dietary α-tocopherol supplementation from 200 to 400 or 500 mg/kg was found (Onibi et al., 2000; Juárez et al., 2011). In addition, Trefan et al. (2011) also reported a gradual decrease in TBARS in conventional pork as levels of dietary α-tocopherol increased up to 100 mg/kg, with no further changes at higher levels of supplementation. On the other hand in subcutaneous fat, levels of 175 mg/kg α-tocopherol had a greater protective effect compared to 95 mg/kg α-tocopherol.

It was expected that the improved antioxidant properties of the subcutaneous fat would have been reflected in an enhanced oxidative stability of the dry fermented sausages, but the higher α-tocopherol levels of the raw materials offered no apparent advantage in reducing lipid oxidation or improving colour stability of the dry fermented sausages. Only the modest differences in colour parameters just after slicing, indicate a slight positive effect of α-tocopherol, with the lower b* values indicating less lipid oxidation in the fat fraction of the dry fermented sausages. Of course, the oxidation reactions in dry fermented sausages are more complex compared to fresh meat and subcutaneous fat, due to factors such as processing procedures and additives used. Mincing and mixing disrupt muscle structure and increase the surface exposed to oxygen and other oxidation catalysts (Chizzolini et al., 1998). The pro-oxidant effect of NaCI seems in part to be attributed to the capability of NaC1 to displace iron ions from binding macromolecules for oxidative reactions (Kanner, 1994), while the curing agents may act as antioxidants (Honikel, 2008). The fermentation process is based on the interaction between meat, fat, bacterial growth, physico-chemical phenomena and biochemical processes. Oxidation of unsaturated fatty acids can occur during maturation and, to a certain extent, it is a useful phenomenon as it produces flavour compounds (Ordóñez et al., 1999).

Doses of 400 mg/kg α-tocopherol in the pig diet are already high and supplementing even higher concentrations would not be useful. Hence, there is a need for other approaches to reduce oxidation processes in n-3 PUFA enriched meat products, for instance, by added antioxidants during processing (Valencia et al., 2007; García-Íñiguez de Ciriano et al., 2009; García-Íñiguez de Ciriano et al., 2010).

CONCLUSIONS

The oxidative stability, in terms of lipid oxidation and colour stability, of n-3 PUFA enriched loin and dry fermented sausage was not improved by supplementing 175 or 400 mg/kg dietary α-tocopherol levels, compared to the supplementation of 95 mg/kg. A beneficial effect of α-tocopherol against lipid oxidation was observed in the subcutaneous fat, but this was poorly reflected in the oxidative stability of the dry fermented sausages.

ACKNOWLEDGEMENTS

It is possible to produce n-3 PUFA enriched meat products that substantially increase the daily recommended nutrient intake of EPA and DHA.

Do these enhanced products improve the health status after consumption?

Part I Lowering the ingoing nitrite dose	Part II Increasing the n-3 PUFA content
CH1 With a dog rose extract	CH5 Role of n-3 PUFA source
CH2 With sodium ascorbate	CH6 Effect on sensory quality
CH3 With a pre-converted extract	CH7 Role of α-tocopherol in feed
CH4 Nitrite and protein oxidation?	**CH8 Effect on health**
CH9 General discussion and future prospects	

DIETS CONTAINING N-3 PUFA ENRICHED PORK: EFFECT ON BLOOD LIPIDS, OXIDATIVE STATUS AND ATHEROSCLEROSIS IN RABBITS

ABSTRACT

The beneficial health effects of n-3 fatty acids are mainly attributed to the very long chain n-3 fatty acids eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-6, DHA). However, in most n-3 enriched food products α-linolenic acid (C18:3n-3, ALA) is used as the source of n-3 fatty acids, assuming that conversion of ALA to EPA and DHA will occur in the body after consumption. In this research health biomarkers were evaluated in rabbits after consumption of pork enriched with n-3 fatty acids originating from linseed oil (ALA) or fish oil (DHA+EPA) fed pigs. Rabbits were fed a pelleted diet simulating a meat-based adult human diet. The meat fatty acid profile was steered by feeding pigs a diet with linseed- or fish oil. Significant changes in the fatty acid profile of different tissues and blood were found in the rabbits. During the experiment, the Total Cholesterol/HDL-Cholesterol (TC/HDL-C) ratio decreased and the TC/LDL-Cholesterol (TC/LDL-C) ratio increased in the fish oil pork group, while for the linseed oil pork group the TC/HDL-C ratio increased and no effect was measured for the TC/LDL-C ratio. The oxidative status was altered by the dietary treatments compared to the baseline and atherosclerosis developed during the experiment, but no differences between the two feeding groups were found. The results highlight the importance of selecting the appropriate n-3 fatty acid source for enriching food products, since pork from fish oil fed pigs differently affected health parameters compared to pork from linseed oil fed pigs.

INTRODUCTION

Health authorities in industrialized countries are nowadays advising to increase the consumption of n-3 fatty acids, particularly the very long chain fatty acids EPA and DHA. The n-3 fatty acids are considered important for normal growth and development and for decreasing or delaying the development of a number of chronic diseases including cardiovascular disease, hypertension and diabetes (Simopoulos, 1999). The main effects of n-3 fatty acids on human health can be divided into three main categories: their essentiality in specific organs, their significant role in lowering blood lipids and their role as precursors for mediating biochemical and physiological responses (Williams, 2000; Ruxton et al., 2004; Narayan et al., 2006). However, besides the claimed positive health effects, these fatty acids are more prone to oxidation due to their highly unsaturated character. Increased oxidation of lipids can disturb the oxidative status of an organism and consequently affect the endogeneous antioxidant enzyme activity of GSH-Px and CAT (Hsu et al., 2001). There is evidence that an altered oxidative status is associated with diseases such as diabetes, Alzheimer's disease and atherosclerosis (Stocker & Keaney, 2005).

Despite attempts to provide education about healthier eating patterns, there are several barriers such as a lack of interest towards changing one's diet, or concerns about having to compromise on taste or enjoyment (Kearney & McElhone, 1999). A more successful strategy to improve the n-3 fatty acid content of the overall diet would be to provide these fatty acids in food products that are already popular. Besides adding n-3 fatty acid sources during the processing of foods, incorporation of n-3 fatty acid rich products like grass, rapeseed, linseed and fish oil in livestock feeds, resulting in accumulation of these fatty acids in animal products, is receiving increasing interest (Raes et al., 2004). Most of these n-3 fatty acid rich products provide ALA as source for n-3 fatty acids, claiming that conversion of ALA to EPA and DHA will occur in the body. However, according to Brenna (2002), only 25% of the administrated ALA is available for the production of very long chain polyunsaturated fatty acids, since the other part is used for

metabolic pathways through β-oxidation. The equal amounts of linoleic acid (C18:2n-6, LA) and ALA in both diets in this study, allows assessing the effects of the very long chain fatty acids versus the effect of enriching feeds with ALA to be converted to longer chain derivatives in the animal. The hypothesis of this study was that the use of fish oil or linseed oil in the pigs feed, results in different fatty acid profiles and consequently affect in a different way the health effects and oxidative status of humans consuming the n-3 enriched meat.

To verify this hypothesis, the objective of this study was to investigate the effects of consumption of dietary n-3 fatty acid enriched pork on the fatty acid composition, oxidative status, blood lipid profile and the development of atherosclerotic lesions. As i*n vivo* animal studies have established that rabbits are susceptible to experimental atherosclerosis induced by feeding a high-cholesterol diet and this animal species shares several aspects of lipoprotein metabolism with humans (Moghadasian, 2002), the rabbit was chosen here as an experimental model.

MATERIAL AND METHODS

1. Experimental set-up

For this experiment 10 male New Zealand rabbits were used. From the age of 8-9 weeks and a mean weight of 2.20 ± 0.12 kg, the rabbits were housed for 8 weeks in individual cages at the Institute for Agricultural and Fisheries Research (ILVO, Melle, Belgium). They were separated in two equal groups (n=5 per group) based on live weight and feed intake. One group received a diet including pork and lard from pigs fed linseed oil (LP group) and the other group a diet with pork and lard from pigs fed fish oil (FP group) (see further). The animals received water *ad libitum* and 140 g feed per day in the morning, in accordance with their requirements for maintenance and growth. Feed refusals were removed daily and weighed. Live weight was

recorded weekly. Blood samples of the rabbits were taken from the ear vein at the start of the trial and after 4 and 8 weeks. The blood was immediately separated into plasma and red blood cells (2000 g, 10 min), and stored at –21°C until analysis. After 8 weeks, the rabbits were euthanized by intravenous injection of T61 (Intervet, Mechelen, Belgium). At necropsy, samples were taken from the heart, coronaries, ascending aorta, thoracic aorta, abdominal aorta and femoral artery. The vessels were fixed immediately in phosphate buffered 10% formaldehyde. The samples were embedded in paraffin according to standard techniques. Heart and part of the thoracic aorta were stored vacuum packed at -21°C for fatty acid analysis. The study was approved by the Ethical Committee of ILVO, Belgium (2005/34).

2. Feed composition

The two experimental diets consisted of conventional rabbit feed ingredients, to which different sources of cooked pig meat and lard were added to simulate a meat-based human diet. The diets were supplemented with 1% of cholesterol to induce atherosclerosis. The pork products were available from a previous trial (Haak et al., 2008), in which the pork fatty acid profile was steered by feeding pigs either a diet with linseed oil (rich in ALA) or fish oil (rich in EPA and DHA). To incorporate the pig meat in the feed pellets, the meat (*longissimus thoracis* muscle) was cooked for 40 min at 70°C, ground (2700 g, 20 s) and lyophilized. Subsequently the lyophilized meat was ground again (22400 g, 20 s). The cholesterol, pig meat powder and melted lard were mixed with the basic feed and pelleted. The ingredient composition of the rabbit feed is presented in Table 8.1. The cholesterol content was not measured in the final diet, but it can be calculated that the contribution of cholesterol from the meat and the lard used in the diets is negligible compared to the 1% added cholesterol. The amount of pig meat and lard included in the feed was calculated based on the human consumption database of Belgium (Devriese et al., 2006). According to these data, the daily intake of an adult is on average 135.0 fresh meat and 43.6 g meat products. Assuming that meat products consist of 50% lard and 50% meat, the total

average daily intake is 156.8 g meat and 21.8 g lard. This daily intake corresponds to 146.6 kcal for the meat fraction (based on a content of 20% protein $\times$ 4 kcal/g protein and 1.5% fat $\times$ 9 kcal/g fat) and 157.0 kcal for the lard fraction (based on 80% fat $\times$ 9 kcal/g fat). Assuming an average daily total energy intake of 2200 kcal, the contribution of meat to the total energy intake was estimated at 6.66% and the contribution of lard at 7.14%. The daily rabbit energy intake was set at 600 kcal, so proportionally 40 kcal (42.8 g) meat and 42.8 kcal (5.9 g) fat, had to be included in the experimental diet. Taking into account the daily allowance of 140 g feed, this corresponded to 30.55% meat (=7.64% lyophilized pork) and 4.25% lard.

Table 8.1. Composition (g/100 g) of the experimental rabbit feed

Composition	Content
Wheat shorts	30.20
Wheat	7.00
Sunflower seed scrap	13.00
Beet pulp	9.00
Flax chaff	21.00
Molasses	4.00
Calcium carbonate	0.20
Mineral-vitamin premix[a]	2.50
Salt	0.11
Methionine	0.10
Lyophilised pork	7.64
Lard	4.25
Cholesterol	1.00
Chemical composition	
Crude fat	9.53
Crude protein	17.60
Crude fibre	14.12
Dry matter	89.62
ME[b](kcal/kg feed)	2476

[a]Konimix 25 Green®:11.9% calcium, 4.4% phosphor, 6.2% sodium, 320 UI/g vitamin A, 70 UI/g vitamin D3, 0.80 mg/g vitamin E, 0.020 mg/g vitamin K3, 0.020 mg/g vitamin B1, 0.11 mg/g vitamin B2, 0.27mg/g calcium-D-pantothenate, 0.020 mg/g vitamin B6, 0.00060 mg/g vitamin B12, 0.71 mg/g nicotinic acid, 4.46 mg/g choline, trace elements;

[b]Metabolisable energy, derived from rabbit feedstuff tables (Maertens et al., 2002). For the enriched meat and lard fraction, the Atwater nutrient conversion factors 4, 9 and 4 kcal/g of carbohydrate, fat and protein respectively were used.

3. Chemical analyses

As a measure of oxidative stability of the feeds the peroxide value was determined, indicating the quantity of reactive oxygen present in the feed. The fat was extracted from the feed with petroleum ether and the peroxide value of the extract was subsequently determined by iodometric titration according to Gray et al. (1978). The amount of iodine that is released by the addition of KI is equivalent with the amount of reactive oxygen present in the sample. The peroxide levels in the feeds were measured after 1 and 8 weeks storage at 7°C and are expressed in meq O_2/g fat.

The lipids from the feed, plasma, red blood cells, heart and thoracic aorta were extracted using chloroform/methanol (2/1; v/v) (modified after Folch et al., 1957). Fatty acids were methylated and analyzed by gas chromatography (HP6890, Brussels, Belgium) on a CP-Sil88 column for FAME (100 m×0.25 mm×0.25 µm; Chrompack, The Netherlands), according to Raes et al. (2001). Peaks were identified based on their retention times, corresponding with standards (NuChek Prep., USA; Sigma, Bornem, Belgium). Nonadecanoic acid (C19:0) was used as an internal standard to quantify the individual and total fatty acids. The fatty acid profiles are expressed in g/100g of total FAME (fatty acid methyl esters) and the total FA content was expressed in mg FA/100g or µg FA/ml.

Plasma total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and triacylglycerols (TAG) were determined using commercial kits (Randox, Crumlin, United Kingdom). The concentrations are expressed in mmol/L.

The ferric reducing/antioxidant power (FRAP) assay for plasma is based on a redox reaction in which an easily reduced oxidant (Fe^{3+}) is used in stechiometric excess and antioxidants act as reductants (Benzie & Strain, 1996). At low pH, the ferric–tripyridyltriazine (Fe^{3+} – TPTZ) complex is reduced to the ferrous (Fe^{2+}) form. The reaction results in an increased absorbance

due to the development of an intense blue chromogen, which was monitored for 20 min at 593 nm and 37°C. FRAP values are expressed in µmol Fe^{2+}/L plasma.

Red blood cells were repeatedly washed with a 0.9% NaCl solution (1000 g, 10min) until colourless. The pellets were used to determine the catalase (CAT) and glutathione peroxidase (GSH-Px) activity.

The CAT activity assay was performed as described by Aebi & Bergmeyer (1974). Diluted red blood cell pellets were mixed with 30 mmol/L H_2O_2 in 50 mmol/L phosphate buffer (pH 7.0) at 22°C. The resulting H_2O_2 decomposition was monitored by measuring the absorbance at 240 nm during the initial 30 s. An extinction coefficient of 40 L/(mol·cm) was used for the calculation. The CAT activity was expressed as µmol H_2O_2 oxidized/(min·ml). The GSH-Px activity was determined by measuring the oxidation of NADPH at 25°C (Hernández et al., 2004). The assay medium consisted of 1 mmol/L reduced glutathione, 0.15 mmol/L NADPH, 0.15 mmol/L H_2O_2, 40 mmol/L potassium phosphate buffer (pH 7.0), 0.5mmol/L EDTA, 1 mmol/L NaN_3, 500 units of glutathione reductase and diluted red blood cell pellets or plasma. Absorbance at 340 nm was recorded over 3 min. An extinction coefficient of 6220 L/(mol·cm) was used for calculation of the NADPH concentration. GSH-Px activity was expressed as µmol NADPH oxidized/(min·ml).

4. Histological and immunohistochemical examination

Paraffin embedded samples were cut at 4 µm and stained with Haematoxylin Eosin, Von Kossa and Von Giesson staining. All arteries were inspected for atherosclerosis by measuring the proportion of the cross sectional circumference covered with plaques. In addition, the thickness of the neointima and media were measured. An overall score (expressed as %) was calculated as follows: (Thickness neointima/(thickness neointima+media))×proportion of the circumference covered with plaques.

5. Statistical analyses

Data concerning blood measurements were analyzed using the general linear model procedure with time (week 4 and 8) and dietary group (FP and LP) as fixed factor and rabbit as random factor. Baseline values were not included in this analysis. Baseline values, calculated across the dietary groups, were compared with the overall mean, calculated across dietary group and time, using a two sample T-test. Data concerning other tissues were analyzed using one-way ANOVA with dietary group as fixed factor. Significance was accepted at $P<0.05$ (SAS Enterprise guide 4).

RESULTS

1. Experimental diets and animal performances

The fatty acid composition of the experimental diets is shown in Table 8.2. There were no differences between the diets, except for EPA, docosapentaenoic acid (DPA, C22:5n-3) and DHA, which were respectively 5-, 3- and 24-fold higher in the FP diet compared to the LP diet. The peroxide values of the FP and the LP diet were 42.8 ± 0.4 and 38.5 ± 0.8 meq O_2/g fat respectively at the start of the trial, and 24.0 ± 2.4 and 21.7 ± 1.1 meq O_2/g fat respectively at the end of the trial. The experimental diets did not affect feed intake (117 ± 12 g/day) and live body weight (3.44 ± 0.37 kg at the end of the trial) of the rabbits.

Table 8.2. Fatty acid composition (g/100g fame) of the two experimental diets

	LP	FP
SFA	26.4	27.7
MUFA	31.0	31.8
LA	25.4	26.0
AA	0.26	0.21
ALA	13.6	13.1
EPA	0.06	0.31
DPA	0.13	0.41
DHA	0.02	0.47
∑ n-6	25.8	26.6
∑ n-3	13.8	14.3
Total FA (mg FA/100g feed)	8511	8042
n-6/n-3	1.87	1.86
PI (%)	56.3	63.1

LP=linseed oil enriched pork group; FP=fish oil enriched pork group;
SFA=C12:0+C14:0+C16:0+C17:0+C18:0+C20:0 +C22:0;
MUFA=C14:1+C16:1+C17:1+C18:1t9+C18:1t11+C18:1c9+C18:1c11+C20:1; LA=C18:2n−6;
AA=C20:4n−6; ALA=C18:3n−3; EPA=C20:5n−3; DPA=C22:5n−3; DHA=C22:6n−3;
∑n−6=C18:2n−6+C18:3n−6+C20:3n−6+C20:4n−6+C22:4n−6;
∑n−3=C18:3n−3+C20:5n−3+C22:5n−3+C22:6n−3; PI=peroxidisability index=(%monoenoic acid*0·025)+(%dienoic acid*1)+(%trienoic acid*2)+(%tetraenoic acid*4)+(%pentaenoic acid*6)+(%hexaenoic acid*8); Kang et al. (2005).

2. Fatty acid profile

Both for red blood cells and plasma, no differences were present between the two feeding groups at the start of the experiment (P>0.05, data not shown). A significant 2 to 3-fold increase of the total fatty acid content in plasma and red blood cells, compared to their baseline values, was observed in both groups during the trial (Table 8.3 and Table 8.4). A significant twofold increase of the total n-3 fatty acids and a small, but significant, increase of the total n-6 fatty acids, compared to their baseline values, occurred due to the administration of the experimental diets, resulting in lower n-6/n-3 ratios in both groups.

Compared with the baseline values, the saturated fatty acid (SFA) proportions significantly decreased, the peroxidizability index (PI), arachidonic acid (C20:4n-6, AA), ALA, EPA and DPA proportions significantly increased and the monounsaturated fatty acid (MUFA) proportions stayed constant in both plasma and red blood cells after administration of cholesterol rich diets. The LA proportion increased significantly in the red blood cells, but stayed constant in plasma, while the DHA proportions significantly increased in plasma and no significant changes were seen in the red blood cells, compared to the baseline values.

During the 8 weeks of experimental feeding, significant treatment effects were observed for both plasma and red blood cells: EPA, DPA and DHA were higher in the FP group compared to the LP group, resulting in a significantly lower n-6/n-3 ratio. In plasma, but not in red blood cells, a significant diet effect was noted for the total n-3 fatty acids and PI, which was higher in the FP group. Also significant time effects were observed for both the red blood cells and the plasma. The total fatty acid content increased significantly between week 4 and week 8, while the total n-3 fatty did not increase in time, probably due to the high ALA proportion that did not change along the feeding experiment. The total n-6 fatty acid content, EPA, DPA, DHA, LA and AA proportions increased significantly in red blood cells between week 4 and week 8. On the contrary, these fatty acids remained constant in plasma, except for EPA which decreased

significantly during the trial. Between week 4 and 8, a significant increase of SFA in the plasma

and a significant decrease of MUFA in the red blood cells was observed.

Table 8.3. Baseline values and diet and time effects on the fatty acid composition (g/100g fame) of plasma

	Baseline[a]	LP[b]		FP[b]		SEM[c]	P-values[d]		
		W 4	W 8	W 4	W 8		P$_{diet}$	P$_{time}$	P$_{interaction}$
SFA	36.2±0.4	30.2	33.5	30.6	33.9	0.45	0.45	<0.001	0.99
MUFA	24.8±0.9	26.2	25.3	26.4	25.5	0.28	0.67	0.15	0.99
LA	26.0±0.8	27.1	26.9	26.6	26.8	0.14	0.35	0.98	0.41
AA	1.36±0.13	2.96	2.84	2.81	2.76	0.12	0.67	0.76	0.90
ALA	3.74±0.15	7.29	6.95	7.01	6.38	0.17	0.23	0.17	0.65
EPA	0.13±0.01	0.24	0.20	0.56	0.49	0.04	<0.001	0.04	0.47
DPA	0.25±0.02	0.78	0.84	1.00	1.19	0.05	0.004	0.12	0.41
DHA	0.11±0.01	0.13	0.18	0.79	0.92	0.08	<0.001	0.05	0.36
∑ n-6	27.7±0.8	30.8	30.3	30.1	30.1	0.33	0.33	0.64	0.55
∑ n-3	4.2±0.1	8.37	8.18	9.37	8.98	0.16	0.01	0.23	0.79
Total FA[e]	1912±153	3400	5287	2995	6108	362	0.68	<0.001	0.18
n-6/n-3	6.6±0.3	3.67	3.73	3.21	3.38	0.08	0.02	0.45	0.72
PI,%	44.1±1.2	63.7	62.5	70.2	70.4	1.08	0.001	0.76	0.67

For description of variables see footnote Table 8.2;
[a]Mean value of both groups ±standard error of the mean (n=10);
[b]Mean values after 4 weeks and 8 weeks of experimental feeding (n=5);
[c]Standard error of the mean (n=20);
[d]P-values from the statistical analysis of the results of week 4 and 8 (baseline values not included);
[e]expressed as µg/ml plasma.

Table 8.4. Baseline values and diet and time effects on the fatty acid composition (g/100g fame) of red blood cells

	Baseline[a]	LP[b]		FP[b]		SEM[c]	P-values[d]		
		W 4	W 8	W 4	W 8		P_{diet}	P_{time}	$P_{interaction}$
SFA	42.2±1.2	32.7	31.4	33.8	32.5	0.43	0.27	0.12	0.99
MUFA	29.1±1.5	32.8	29.8	32.7	30.2	0.41	0.83	<0.001	0.55
LA	16.0±0.6	22.2	24.2	20.7	23.2	0.42	0.10	0.01	0.66
AA	2.28±0.20	3.00	3.72	3.00	3.35	0.13	0.49	0.03	0.38
ALA	2.73±0.18	5.84	5.77	5.59	5.34	0.15	0.30	0.63	0.77
EPA	0.13±0.02	0.20	0.22	0.35	0.49	0.03	<0.001	0.03	0.08
DPA	0.40±0.03	0.71	1.17	1.04	1.48	0.07	0.002	<0.001	0.97
DHA	0.53±0.05	0.12	0.29	0.41	0.73	0.06	<0.001	<0.001	0.12
∑ n-6	19.0±0.8	25.9	28.7	24.3	27.2	0.48	0.05	0.002	0.94
∑ n-3	3.79±0.12	6.86	7.44	7.38	8.04	0.16	0.08	0.05	0.89
Total FA[e]	424±62	1017	1235	916	1442	67	0.64	0.01	0.19
n-6/n-3	5.09±0.33	3.81	3.88	3.30	3.39	0.09	0.01	0.52	0.93
PI, %	41.1±1.6	55.5	64.7	58.4	67.8	1.31	0.08	<0.001	0.96

For description of variables see footnote Table 8.2;

[a]Mean value of both groups ±standard error of the mean (n=10);

[b]Mean values after 4 weeks and 8 weeks of experimental feeding (n=5);

[c]Standard error of the mean (n=20);

[d]P-values from the statistical analysis of the results of week 4 and 8 (baseline values not included);

[e]expressed as µg/ml rbc.

In the aorta and heart no effects were seen for the SFA, LA and ALA proportions (Table 8.5). In the heart a higher amount of MUFA and lower amount of AA was detected in the FP group compared to the LP group, whereas in the aorta no effects were observed. The fatty acid profile of the aorta was clearly affected by the diet: EPA, DPA and DHA proportions were 2- to even 5-fold higher in the FP group. In the heart the EPA and DHA proportions were respectively a 1.4- and 3-fold higher in the FP group compared to the LP group, while on the contrary the DPA proportion was higher in the LP group compared to the FP group. The total n-3 fatty acids were significantly higher and the total n-6 fatty acids significantly lower in the heart of FP group compared to the LP group, resulting in a lower n-6/n-3 ratio, but this was not the case in the aorta. The PI was not affected in the aorta or heart. The total fatty acid content was 2.7-fold higher in the aorta of the LP group compared to the FP group, while in the heart no significant difference in total fatty acid content was observed.

Table 8.5. Fatty acid composition (g/100g fame) of aorta and heart

	Aorta				Heart			
	LP	**FP**	**SEM**	**P-value**	**LP**	**FP**	**SEM**	**P-value**
SFA	26.8	26.2	0.43	0.48	24.7	25.4	0.47	0.51
MUFA	32.9	33.7	0.48	0.45	24.9	27.3	0.54	0.01
LA	23.0	21.2	0.62	0.15	23.6	23.6	0.40	0.99
AA	1.07	1.66	0.26	0.28	11.2	7.96	0.72	0.01
ALA	9.36	7.68	0.78	0.31	4.61	5.05	0.27	0.44
EPA	0.11	0.27	0.32	0.003	0.36	0.49	0.03	0.01
DPA	0.38	0.63	0.07	0.03	1.72	1.30	0.01	0.03
DHA	0.07	0.34	0.05	<0.001	0.55	1.73	0.20	<0.001
$\sum$ n-6	24.5	23.4	0.42	0.22	35.6	32.2	0.81	0.03
$\sum$ n-3	9.92	8.91	0.71	0.51	7.24	8.58	0.30	0.01
Total FA[a]	234	87.9	36.4	0.04	2911	3606	184	0.05
n-6/n-3	2.61	2.66	0.15	0.87	4.97	3.78	0.27	0.02
PI,%	51.6	53.9	1.31	0.42	97.6	92.9	2.35	0.34

For description of variables see footnote Table 8.2. Data were analysed using one-way ANOVA with dietary group as fixed factor (P<0.05);
[a]expressed as mg/100g tissue.

3. Blood lipids

No differences were found at the start of the experiment between the two feeding groups (P>0.05, data not shown). Due to the high cholesterol content of the experimental feeds, the values of TAG, TC, TC/HDL-C and LDL-C, but not HDL-C, were significantly higher at week 4 and week 8 compared to the baseline values, while TC/LDL-C was significantly lower (Table 8.6). During the feeding trial the overall concentration of TAG in plasma increased significantly between week 4 and 8. Both TC/HDL-C and TC/LDL-C showed a significant time×treatment interaction. TC/HDL-C ratio decreased and TC/LDL-C increased during the trial for the FP group, while in the LP group TC/HDL-C increased and TC/LDL-C remained constant.

4. Oxidative status parameters

No differences were found between the two feeding groups at the start of the experiment (P>0.05, data not shown). The rabbits responded to the experimental diets with increased plasma FRAP values and decreased GSH-Px activity compared to the baseline values (Table 8.6). In red blood cells, no differences for the GSH-Px activity and higher values for the CAT activity were found following administration of cholesterol rich diets. During the trial, the GSH-Px activity of plasma was higher at week 8 compared to week 4, irrespective of the feeding group.

Table 8.6. Baseline values and diet and time effects on plasma lipid and oxidative status parameters

	Baseline[a]	LP[b]		FP[b]		SEM[c]	P-values[d]		
		W 4	W 8	W 4	W 8		P_{diet}	P_{time}	$P_{interaction}$
TAG[e]	1.16±0.05	1.84	3.81	1.01	3.01	0.44	0.33	0.04	0.98
TC[e]	2.26±0.11	29.3	39.6	27.5	22.9	3.26	0.23	0.62	0.21
HDL-C[e]	1.07±0.08	0.98	0.82	0.57	1.09	0.10	0.71	0.35	0.09
TC/HDL-C	2.20±0.16	33.4	55.6	49.7	24.5	5.13	0.52	0.84	0.01
LDL-C[e]	0.67±0.11	27.5	37.1	26.5	20.4	3.06	0.24	0.74	0.16
TC/LDL-C	5.14±1.43	1.07	1.08	1.04	1.13	0.01	0.55	0.03	0.04
FRAP[f]	377±23	682	908	707	709	61.5	0.57	0.30	0.31
GSH-Px[g], plasma	0.58±0.02	0.422	0.505	0.415	0.521	0.02	0.90	0.03	0.76
GSH-Px[g], rbc	0.15±0.01	0.248	0.192	0.259	0.234	0.03	0.71	0.55	0.82
CAT[g]	298±51	1463	1561	1409	1897	171	0.75	0.32	0.50

LP= linseed oil enriched pork group; FP= fish oil enriched pork group; Wk = week; TAG = triacylglycerol, TC = total cholesterol, HDL-C = HDL-cholesterol; LDL-C= LDL-cholesterol, FRAP= ferric reducing/antioxidant power; GSH-Px = Glutathione peroxidase; CAT = catalase; rbc = red blood cells, U= µmol NADPH oxidized/min for GSH-Px and µmol H_2O_2 oxidised/ min for CAT;

[a]Mean value of both groups ±standard error of the mean (n=10);

[b]Mean values after 4 weeks and 8 weeks of experimental feeding (n=5);

[c]Standard error of the mean (n=20) ;

[d]P-values from the statistical analysis of the results of week 4 and 8(baseline values not included);

[e] expressed as mmol/L;

[f] expressed as µmol Fe^{2+}/L plasma;

[g] expressed as U/ml.

5. Atherosclerosis

All rabbits in both groups did present atherosclerotic lesions that varied in extent of affected area from minimal to severe (Figure 8.1), but no significant differences were seen between the groups. For the coronaries, femoral artery, ascending, thoracic and abdominal aorta a mean atherosclerotic plaque deposition of 14.0±17.6, 15.8±10.7, 36.4±14.1, 29.7±16.0 and 24.0±10.8% respectively was found. Some small cardiac arteries had a thickened, eosinophilic tunica media with associated narrowing of the lumen. Examination of the ascending, thoracic and abdominal aorta revealed lesions consisting of thickening of the tunica intima by the presence of foam cells, smooth muscle cells/myofibroblasts, extra cellular lipids, lymphocytes and macrophages and fibrous cap formation.
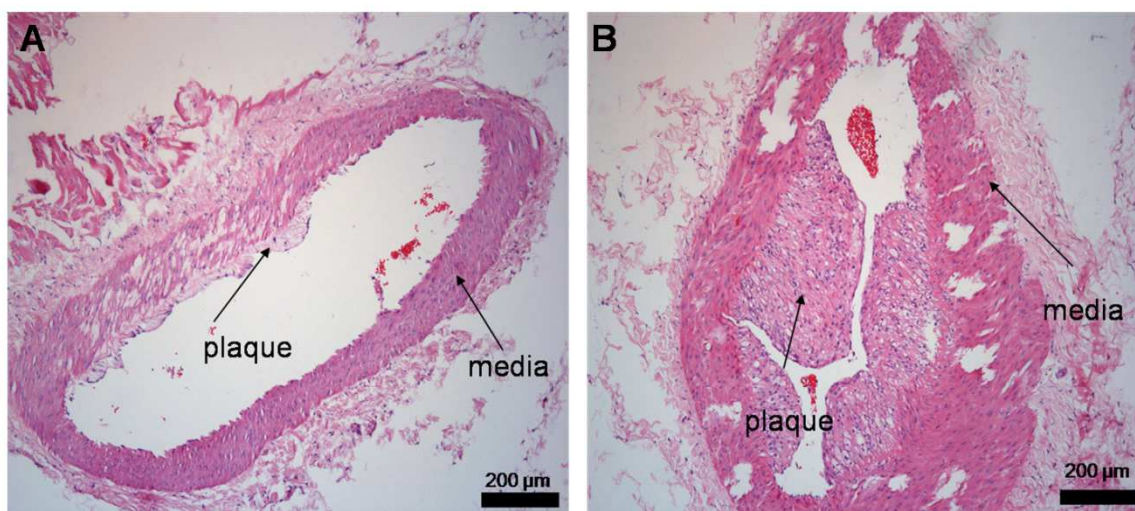
Fig 1. Example of Haematoxylin Eosin, Von Kossa and Von Giesson staining for evaluating the atherosclerosic plaque deposition among groups. An overall score (expressed as %) was calculated: (Thickness neointima/(thickness neointima+media))×proportion of the circumference covered with plaques. Figure 1A shows mild atherosclerosis in the coronary (overall score of 8.3%), while figure 1B represents severe atherosclerosis in the femoral artery (overall score of 48%).

DISCUSSION

In the experimental diets, next to the enriched pork, also the enriched lard of the same pigs was used. Lard contains less long chain n-3 polyunsaturared fatty acids compared to muscle, explaining why the proportions of EPA and DHA are lower in the experimental diets compared to the added pork, originating from Haak et al. (2008). For the FP group, the lard most probably contained slightly more DHA than EPA, which consequently altered the ratio EPA/DHA in the diet compared to the ratio of the meat reported in Haak et al. (2008). Analysis of the fatty acid composition of the feed revealed a daily consumption of 8.3 mg EPA and DHA for the LP group and 71.1 mg EPA and DHA for the FP group. These concentrations represent 0.02% of the daily total energy intake (E) for the LP group and 0.25% E for the FP group. The Superior Health Council of Belgium (2006) recommends for healthy humans that at least 0.3% E should originate from EPA and DHA. Hence, the recommended level was approached in the FP group, but an additional intake of EPA and DHA from other sources is required for the LP group. According to Burdge (2004), conversion percentages of ALA to EPA vary between 0.2% and 8%. The extent of conversion of ALA to DHA is less clear. The highest estimated fractional conversion is 4%, while others have either failed to detect significant DHA synthesis or estimated that less than 0.05% of ALA was converted to DHA (Burdge, 2004). The latter was also observed in our results, showing no enhanced DHA proportions in plasma and red blood cells of the LP group compared to their baseline values. Consequently, enrichment of foods with ALA is not as effective as enrichment with EPA and DHA to enhance the long chain fatty acid pool in the body. It should be mentioned that in the present study both diets contained similar levels of ALA, hence the FP diet supplied more EPA and DHA in addition to the same supply of ALA. The major source of ALA in the diets was flax chaff, which was added to meet the absolute dietary requirement of rabbits for sufficient fibre. As a result, the effects observed in the present study for the FP diet cannot be exclusively ascribed to the higher supply of EPA and DHA, but might

be a combined effect of the supply of all n-3 fatty acids. The differential effects between the LP and the FP diet must however originate from the very long chain n-3 fatty acids. Additional experimental groups fed on a low total n-3 diet, and a low ALA – high EPA and DHA diet would have been interesting and should be envisaged in future research aiming at investigating the potential of enriched meats.

Mantzioris et al. (2000) showed that, when consuming n-3 enriched food (ALA or DHA and EPA), the concentrations in red blood cells of healthy adults were changed after 2 weeks, thereby indicating the relatively short time required for n-3 fatty acid concentrations to rise. In the present study, the n-6/n-3 ratios in plasma and red blood cells were significantly lowered after administration of the n-3 fatty acid enriched diets, meaning that the n-3 fatty acids were absorbed and incorporated in red blood cell membranes and that the meat matrix did not inhibit the absorption of these fatty acids. The significantly different fatty acid profiles in the heart and aorta between the two feeding groups in the present study confirm this. In a study of Coates et al. (2009) significant effects in humans consuming n-3 fatty acid enriched pork were found. The subjects consumed daily about 0.7% E long chain n-3 fatty acids (mainly DHA). DHA levels rose 15% in red blood cells of the n-3 group, whereas they decreased by 5% in the control group that consumed non-enriched pork. In addition, serum TAG decreased to a greater extent in the n-3 group. In most supplementation studies in which beneficial effects on the blood lipid profile were achieved, 1 g/day (about 0.4% E) EPA and DHA or more was administered (Lovegrove et al., 1997; Garg et al., 2007). It is remarkable that in the present study, with lower intakes of EPA and DHA, a significant decrease of TC/HDL-C and increase of TC/LDL-C was found in the FP group. Deckelbaum et al. (2008) stated that habitual consumption of n-3 fatty acids may be more beneficial than short-term consumption. Importantly, n-3 fatty acids need not to be considered as a monotherapy in prevention of and/or treatment for cardiovascular disease. Rather, higher n-3 fatty acid intakes need to be considered as a biologically active partner to lifestyle changes.

The PI represents the degree of unsaturation of the lipids and can be used as an indicator of peroxidation susceptibility. In the plasma, significantly higher PI values were found in the FP group compared to the LP group. However, no significant differences were found between the two feeding groups for the oxidative status parameters in the plasma, meaning that diets enriched with very long chain fatty acids do not affect the oxidative status in a different way compared to diets enriched with long chain fatty acids. In concordance, no differences in atherosclerosis were observed. On the other hand, clear responses were observed following cholesterol feeding in the experimental diets. The total fatty acid content of the plasma and RBC increased with time probably due to the high cholesterol content of the feed. Similar results in rabbits were found by Mahfouz & Kummerow (2000) and Risé et al. (2004). Mahfouz & Kummerow (2000) attributed the increase in total fat content to the increased hepatic secretion of β-very low-density lipoproteins (β-VLDL), which is inherent to hypercholesterolemia. These β-VLDL's are remnants derived from mutant chylomicrons and very-low-density lipoproteins that cannot be metabolized completely and accumulate in the blood.

Similar to Alipour et al. (2006), FRAP values increased after the administration of a cholesterol-rich diet. These authors suggested that more antioxidants could be circulating in the rabbits plasma as a response to the cholesterol. On the contrary, Lecumberri et al. (2007) did not observe any difference in FRAP values between normocholesterolemic and hypercholesterolemic rats. Also the higher fat and increased unsaturated fatty acid content of both the FP and LP diets could have enhanced the FRAP levels. However, studies investigating the effect of fat and/or unsaturated fatty acids on plasma FRAP levels have reported inconsistent results (rabbit, Zheng et al., 2006; rat, Walczewska et al., 2010; human, McAnulty et al., 2010). An increased red blood cell CAT activity was observed during the time of the experiment, while the GSH-Px activity in red blood cells remained constant. In a study of Mahfouz & Kummerow, (2000) an increased CAT activity and decreased GSH-Px activity was seen after 2 months feeding a basal diet plus 1%

cholesterol to rabbits, implying an adaptation of CAT, as a response to the failure of GSH-Px. Shull et al. (1991) reported a 3-fold increase of CAT mRNA after addition of the oxidant H_2O_2 (250 µmol/l), whereas the increase of GSH-Px mRNA was less striking. Our results are in accordance with these studies, although no change in GSH-Px activity was found in red blood cells. It seems that the activity of GSH-Px does not respond in a similar way in plasma as in red blood cells, since GSH-Px activity in plasma first decreased and then increased again, which suggests that the organism adapted to its oxidative environment.

CONCLUSIONS

The blood lipid profile response in rabbits to feeding cholesterol was slightly improved in a diet containing pork enriched with EPA and DHA compared to pork enriched mainly in ALA. The fatty acid profile of blood and different tissues, but not the plasma oxidative status and atherosclerosis development, was affected by the source of dietary n-3 fatty acid supply.

ACKNOWLEDGEMENTS

CHAPTER 9

GENERAL DISCUSSION AND FUTURE PROSPECTS

GENERAL DISCUSSION

"Healthy natural food"... the new concept of the 21st century...Although often used for commercial purposes, it is widely accepted that a balanced diet contributes to a healthier life.

While people may be aware of main nutrition messages, they do not perceive these as personally relevant to themselves (Kearney & McElhone, 1999). Therefore, enhancing food products that are already popular can improve the health status of the population, without changing the consumption pattern and, for some cases, without the risk of overdosing. The aim of this PhD research was to contribute to this approach by producing healthier meat, namely by lowering the ingoing nitrite content or increasing the n-3 PUFA content, without compromising its sensory quality.

Meat curing is a preservation technology that has been practiced for centuries and can be traced back to 3000 B.C. People found that salt contaminated with potassium nitrate resulted in meat with an appealing pink colour. Only later it was discovered that actually nitrite, which was accidentally formed from the conversion of nitrate by micro-organisms, was responsible (Honikel, 2008). Although we were not able to repeat this observation by adding nitrate from a *Rosa canina* L. extract directly to frankfurters, probably due to the low nitrate-reducing ability of the natural flora of the meat, this story illustrates how little nitrite is needed to produce a desired pink colour. For example, in liver pâté only 20 mg/kg nitrite was needed to have colour formation **(H1 rejected)** and it should be further explored if even lower concentrations can be used.

Yet, not only colour formation, but also its stability is of great importance. In the present dissertation colour stability was investigated by exposing the samples to light and air at a controlled temperature of 4°C (chilled display conditions). However, many other factors can influence the colour stability of the meat and meat products. To start with, animal and production

factors, such as animal genetics, muscle biochemistry, nutritional background and pre-harvest stress, have an effect on the oxidative status of the raw materials. Also the chilling conditions (e.g. temperature, carcass size and chilling × pH interaction) and post-mortem age of the meat have an influence. In minced meat products the disruption of tissue also affects the colour and increases the oxidative susceptibility due to the release of pro-oxidative compounds such as iron. Further, the processing conditions with mainly the processing ingredients, pH and heating settings have large effects on colour stability of the meat products. The above mentioned processing conditions, except for the processing ingredients, were controlled and kept constant in all samples to be sure that the differences in oxidative stability were caused solely by the factor that was investigated. In addition, all samples were prepared from the same batch of raw materials to exclude animal-depend variations. A primary objective of display studies is to evaluate colour deterioration (or maintenance) over a given time. The quantification of this discolouration is generally not done by fitting the data to a mathematical model. Instead, colour parameters at specific end-points are compared, ignoring the processes between the initial colour and the end-point. As shown in this dissertation, the rate of discolouration can be derived from a mathematical model, given that enough measurements are performed over a certain time frame. The rate of discolouration, which represents the colour stability, can be subjected to statistical analysis, making this approach a potential tool to investigate different factors influencing the colour stability. In addition, as absolute colour values may vary between laboratories, using discolouration rates makes it possible to compare results in a more standardized way.

Lowering the ingoing nitrite to 40 mg/kg resulted in inferior colour stability in liver pâté (**H2 accepted),** which, on the other hand, was not the case with 20 mg/kg nitrite originating from a pre-converted plant extract. It is likely that other compounds present in the pre-converted extract increased the colour stability **(H5 accepted for colour stability)**.

For many years already, researchers are trying to reproduce the characteristic pink colour without the use of nitrite. Colourants mimicking the pink meat colour are available on the market, however, the resulting meat product looks rather artificial. Pegg & Shahidi (1997) produced the nitrosomyochromogen *in vitro*, but its application in commercial meat products lacks, probably because the stability of this preformed cooked cured meat pigment was limited when light and oxygen were present. More recently, Zinc-porphyrine, a red pigment from dry cured ham (Wakamatsu et al., 2004), is studied for its potential use in other cured meat products. However, little success is reported until now. Consequently, one could question whether it is feasible to add very small amounts of nitrite to produce the cured colour and improve the decreased colour stability with other compounds. For example, it is possible to produce cooked porcine patties with 10 mg/kg ingoing nitrite, which results in a product showing the characteristic pink colour with residual nitrite content similar to that of uncured patties (unpublished data).

Nitrite is a powerful antioxidant against lipid oxidation, but various alternatives were found to replace this property of nitrite. Lowering the nitrite content in frankfurters and liver pâté clearly resulted in higher TBARS and volatile compounds (**H3 accepted**), however the *Rosa canina* L. extract, pre-converted extract and sodium ascorbate were able to counteract this effect (**H5 accepted for lipid oxidation**). Less clear is the effect of lowering nitrite on protein oxidation. Positive controls tended to be less susceptible to protein oxidation, but interfering factors hindered us to elucidate the precise role of nitrite against protein oxidation. Moreover, even when conducting a more in depth study, still contradicting results between isolated myofibrillar proteins and raw porcine patties were found **(H4, H6 and H5 for protein oxidation undecided).** Possible reasons for these differences are given in the discussion part of chapter 4, however, a critical point that should not be ignored is that most of the times only carbonyl compounds were analysed. The main reason why this parameter was used, is that carbonyl groups arise from a variety of oxidative processes and consequently provide a generalized or integrated assessment

of oxidative damage (Stadtman & Levine, 2000). Still, other muscle protein oxidation parameters, e.g. cross-linking and fragmentation (Lund et al., 2011), should be further explored to have a broader view on the role of nitrite on protein oxidation.

In this context, 3-nitrotyrosine, a marker extensively used for nitroxidative stress *in vivo* (Souza et al., 2008) was introduced for meat products. There are many parallels between biological processes *in vivo* and meat, e.g. lipid oxidation results in harmful oxidation products, which can induce both meat deterioration and disease. The occurrence of protein oxidation in biological systems has been known and studied for about 50 years because of the relation between the oxidative damage to proteins and the development of disease (Shacter, 2000). On the contrary, the discovery that muscle proteins were susceptible to oxidative reactions leading to potential deleterious effects on meat quality lasts for only 20 years (Estévez, 2011). As a result, many (if not all) markers used for protein oxidation in meat science originate from biomedical research. In general, these protocols are developed for purified proteins or single amino acids and are not adequate for complex matrices. When adapting a method to suit meat analysis, this should not be ignored. In this dissertation, the DNPH method was frequently used to determine protein carbonyl compounds in meat products. Although this method is often applied in meat research, I want to emphasize that caution is needed. To start with, oxidized proteins have different solubility compared to non-oxidized ones, so carefulness is required when proteins are extracted from the matrix. I used a buffer containing 0.6 M NaCl to increase the solubility, but this is not always done in other studies investigating meat quality. In addition, interferences with lipid carbonyl compounds are possible and extensive washing with ethanol:ethyl acetate is mandatory, especially for meat products with high fat content. Finally, the determination of the protein content measuring the absorbance at 280 nm gave in my studies impossible results for liver paté, which is the reason why I used the crude protein content (Kjehldahl method) to express the carbonyl content per mg of protein. Taking into account al these issues, I have some doubts

whether this method is correctly used in meat research. Although many interesting and relevant findings about the effect of protein oxidation on the quality of meat are found in numerous studies, the large variations among replicates that are often reported point out that more caution is needed when adopting a method from another research field. In this view, the use of 3-nitrotyrosine as a marker for protein nitration in meat products, should be approached with the same care. Maybe this partly explains why it is not yet clear whether 3-nitrotyrosine is a good marker for meat products or not (**H7 undecided**).

Amusingly, much of the biochemistry of nitrite has been understood for decades in meat science, but has only recently been rediscovered in human physiology. Research within the biomedical science community has revealed therapeutic benefits of nitrite that is currently being developed as novel therapies for conditions associated with NO insufficiency (Parthasarathy & Bryan, 2012). Without ignoring the fact that meat and especially cured meat products contribute to an increased risk of cancer development (World Cancer Research Fund, 2011), we should revise our long-standing view that nitrate and nitrite are only harmful substances in our diet. In fact, recent studies found a protective effect of nitrite against lipid and protein oxidation during *in vitro* digestion of cured meat model products (Van Hecke et al., 2014).

There is a great interest in improving the fatty acid profile of food to better meet nutritional recommendations. This work showed that the n-3 PUFA content of pork products can be significantly increased by adding moderate levels (0.3-1.2%) of an n-3 PUFA source to the diet. As EPA and DHA are believed to be the bioactive compounds regulating a wide range of biological functions, the main focus in this work was to increase the EPA and DHA content of the products. The addition of linseed oil to the feed resulted in lower EPA and DHA concentrations in the meat products compared to those originating from pigs fed fish oil and microalgae **(H8 accepted)**. Similar to humans, the conversion of ALA to EPA in pigs is low,

pointing out the importance of using adequate sources for EPA and DHA in the feed. The little conversion of ALA to EPA that was found in the present dissertation resulted in a nutritional significant increase of EPA when comparing to soybean oil. However, it is important to note that no changes of DHA concentrations in the meat products were observed, while with fish oil and microalgae both EPA and DHA were substantially enhanced.

Many fish species have been fished almost to extinction and we are on course to eliminate the world's supply (Brunner et al., 2009), so we are urged to seek for alternatives. Microalgae are easy to grow and after separation of the n-3 PUFA from the microalgae lipids, the excessive lipids can be used for biodiesel production, while the biomass can find uses as valuable protein-rich animal feed (Adarme-Vega et al., 2012). If the production of microalgae is carried out at a large scale this would address three major areas of importance: human health, transportable energy and food security. Hence, it can be concluded that microalgae are a sustainable source of EPA and DHA in pig feed, without pressuring the marine ecosystem and therefore a suitable alternative to fish oil (**H9 accepted**). Another sustainable strategy to increase the EPA and DHA content through the pigs diet could be plants high in stearidonic acid (C18:4n-3), such as primrose, echium and hempseed (Lenihan-Geels et al., 2013). The basis of this proposal lies in the ability to bypass the rate limiting enzyme, $\Delta 6$-desaturase, as stearidonic acid is the first desaturation product in the conversion of ALA to its long-chain derivatives. However, Kitessa et al. (2012) presented data to suggest that echium oil has no advantage over linseed oil in enhancing n-3 PUFA in lamb tissues. Likewise, Tanghe et al. (2013) concluded that echium oil at a 1% dose in pig feed had no benefit over linseed oil in increasing the EPA and DHA concentration in the tissues.

Recently, transgenic plants and animals have been designed with increased EPA and DHA content (Venega et al., 2010; Houdebine, 2014), however, the use of these products for human consumption in the near future is not very likely due to the consumer aversion to genetically

modified food. On the other hand, these transgenic enriched plants can be added to animal feed as genetically modified plants are already commonly used as feedstuff (Aumaitre et al., 2002).

The major part of a pig carcass is processed to various meat products, while a smaller part is used for fresh meat cuts. As the composition and processing techniques influence the oxidative stability of a meat product, it is relevant to not only asses the impact of supplementing n-3 PUFA in fresh meat, but also in processed products. This is of interest when commercialising entire carcasses of pigs fed an n-3 PUFA enriched feed. The oxidative susceptibility of n-3 PUFA enriched meat products was increased (**H10 accepted**), while the stability of the fresh loin was little affected. Loin is consumed shortly after production, has a relatively low fat content and no processing is involved, whereas dry cured ham and dry fermented sausages are exposed to a more oxidative environment, e.g. long ripening periods and mincing, which influences the oxidative stability. In further research, also cooked meat products should be taken into account, as heating can induce oxidation. Also, these products have generally a high fat content, which obviously can lead to more lipid oxidation products, similarly to what was observed in the dry fermented sausages. The advantage of meat products over fresh meat is that off-flavours can be reduced or even masked by the addition of antioxidants or other compounds such as spices during manufacturing. Though, this implies that for all meat products new recipes should be developed, which is time-consuming and has a cost. Of course, a solution could be not to produce meat products highly susceptible to oxidation, but this would imply that the n-3 PUFA enriched carcasses can not be processed simultanously with the conventional carcasses. Moreover, these susceptible meat products generally have a high fat content and would therefore contribute more to the daily intake of EPA and DHA.

Another approach is to add antioxidants in the pig feed, which in addition could benefit the oxidative status of the feed, the animal, fat tissue and unprocessed fresh meat. In this dissertation,

however, we did not succeed to improve the oxidative stability of the pork products with increasing dietary α-tocopherol levels from 90 to 400 mg/kg (**H11 rejected**).

As the sensory quality of enriched products greatly depends on the absolute EPA and DHA content, the focus should not be on extremely increasing the EPA and DHA content of one food product, but on increasing a number of widely consumed food products with moderate amounts of EPA and DHA. Consider Table 9.1 as an example. The average daily meat intake was based on the human database of Belgium (Devriese et al., 2006) with 135 g/day fresh meat and 43.6 g/day processed meat. For this example it was assumed that all fresh meat was pork loin and meat product intake consisted of 50% dry fermented sausages and 50% dry cured ham. Daily EPA and DHA intake was calculated using the EPA and DHA content of the meat products from chapter 5 (Table 5.7). The conventional, moderate and high diets were calculated from respectively the SOY, ALG LOW and ALG HIGH groups. As the dry fermented sausages of the ALG HIGH were sensory unacceptable, the conventional product was used for the calculations. Similar amounts of EPA and DHA were consumed in both enriched diets, but with the moderate diet, problems with oxidative stability and sensory quality are omitted and no economical losses from useless carcass parts should be taken into account. When consuming these moderate enriched products, about 54 mg EPA+DHA is ingested and this corresponds with 8% of the daily recommeded intake. Moreover, four fold higher intakes were achieved when compared to a conventional diet.

Table 9.1. Contribution of n-3 PUFA enriched meat products to a healthier diet

	Average intake (g/day)[1]	EPA+DHA intake (mg/day)[2]		
		conventional	moderate	high
Fresh meat	135	6	18	39
Dry fermented sausage	21.8	5	30	5[*]
Dry cured ham	21.8	2	7	16
Total		*14*	*54*	*60*
%RNI[3]		*2*	*8*	*9*

*As the dry fermented sausages were sensory unacceptable, the conventional product was used;

[1]Based on the human database of Belgium (Devriese et al., 2006);

[2]Based on Table 5.7, conventional = SOY, moderate = ALG LOW and high = ALG HIGH.

[3]%RNI was calculated with a daily recommended nutrient intake of 667 mg EPA+DHA (=0.3% of the total daily energy intake).

As meat is one of the main sources of long chain n-3 PUFA, apart from fish and seafood (Howe et al., 2006), even moderate improvements of their fatty acid composition are valuable. In this view, it is more of interest to enrich meat through animal feeding, with the production of various n-3 PUFA enriched meat products, instead of adding n-3 PUFA during processing, which results in only one kind of enriched product. In fact, increasing the n-3 PUFA content of meat needs to be part of the global strategy to increase the total daily intake through various enriched food products.

Studies on improving the fatty acid composition of meat usually do not further investigate the consumption of these enhanced products. Yet, that is the key question: does the n-3 PUFA enriched meat truly contribute to a healthier life?

In chapter 8, n-3 PUFA enriched meat was incorporated into the diet of a rabbit, used as a model for humans. The EPA and DHA content of the plasma and other tissues was higher when consuming meat from pigs fed fish oil, compared to meat origination from pigs fed linseed oil, again showing the importance of directly consuming EPA and DHA (**H12 accepted**). Improved

cholesterol parameters were found in the EPA and DHA enriched diet, but not in the ALA enriched diet (**H13 accepted**). From this, we should reconsider food products presently available on the market enriched with an ALA source, as they marginally contribute to an increased intake of EPA and DHA. There is a need to shift the production of (meat) products enriched with ALA towards the production of EPA and DHA enriched products.

To conclude, dietary adjustments may not only influence the present health, but may determine whether or not an individual will develop diseases much later in life.

FUTURE PROSPECTS

In the general discussion several issues that deserve further consideration and research came forward:

   i.   Only small levels of nitrite are necessary to have proper colour formation. However, these levels will depend on the properties of the meat product (e.g. fat and meat content, processing conditions), meaning that the adequate nitrite level has to be determined separately for all kinds of meat products. In this context, also suitable active compounds to maintain the colour stability have to be searched for product-specifically.

   ii.   The role of nitrite during protein oxidation remains to be elucidated. Other levels of nitrite could be tested using the same experimental set-up of chapter 4. It is also advised that additional protein oxidation parameters (e.g. thiol groups and tryptophan depletion) are assessed.

   iii.   The marker 3-nitrotyrosine was present in meat, but apart from this the function of 3-nitrotyrosine in meat products and in general the effect of protein and lipid nitration on meat quality remains to be elucidated.

   iv.   Statements concerning the effect of nitrite intake on human health are contradictory and further studies to clarify these contrasting effects are necessary.

   v.   Microalgae are a useful direct source of EPA and DHA in pig feed, but studies investigating the sensory quality of the resulting n-3 enriched meat products are scarce. For instance, the effect of cooking and preservation should be further investigated, especially for meat products with high fat content.

   vi.   The most cost-effective level of microalgae in pig feed has to be determined. This level should result in meaningful levels of n-3 PUFA in meat that at the same time does not need special attention during processing and that can be used the same way as

conventional pork. From this dissertation it was found that this level will be between 0.3 and 1.2 g algae/100g feed.

vii. A wide range of natural dietary antioxidants are available on the market and it could be explored whether these can be applied in pig feeds to increase the oxidative stability of n-3 PUFA enriched meat products. Importantly, a main aspect that has to be considered is whether the antioxidant is incorporated in muscle tissue.

viii. There is a need to shift the production of (meat) products enriched with ALA towards the production of EPA and DHA enriched products. If all food products, that contain a relevant amount of fat, are moderately enriched with EPA and DHA the recommended daily intake of EPA and DHA could be achieved.

SUMMARY

It is well established that inadequate dietary habits and physical inactivity are the major preventable risks for the occurrence of chronic diseases. Besides providing education about healthier eating patterns, improving the composition of widely consumed food products is an alternative approach. The aim of this dissertation was to investigate the effects of lowering the ingoing dose of potentially harmful nitrite, by replacing it with other compounds in cooked meat products (Chapter 1-4) and to increase the content of health-promoting n-3 polyunsaturated fatty acids (n-3 PUFA) in pork products through animal feeding (Chapter 5-8). The overall objective was to change the composition without compromising the oxidative stability and sensory quality, focussing on colour, lipid and protein oxidative processes.

In chapter 1 the effect of a dog rose extract (*Rosa canina* L.) in nitrite-free frankfurters was investigated. Dog rose is a source of nitrate, polyphenols and ascorbic acid. Frankfurters were produced with 5 or 30 g/kg dog rose extract, without the addition of sodium nitrite or sodium ascorbate. The frankfurters with dog rose extract showed similar lipid oxidation compared to a positive control containing 100 mg/kg sodium nitrite and 500 mg/kg sodium ascorbate. The dog rose extract protected against protein oxidation, but not as efficiently as the positive control. As the dog rose treated frankfurters showed inferior colour traits compared to nitrite-treated frankfurters, it was concluded that dog rose could act as a natural antioxidant in frankfurters, but not as a full replacer for sodium nitrite.

Chapter 2 deals with the effectiveness of partly replacing sodium nitrite by sodium ascorbate for its antioxidant activity in liver pâté. The effect of sodium ascorbate (500, 750, 1000 mg/kg) and sodium nitrite (40, 80, 120 mg/kg) dose on the shelf-life stability was assessed in a full factorial design. Decreasing the nitrite dose to 80 mg/kg had no negative impact on colour formation, colour stability and lipid oxidation. No additional antioxidant effect of sodium ascorbate was

noticed. Lowering sodium nitrite to 40 mg/kg resulted in proper colour formation, but the colour stability was inferior and lipid oxidation increased. At this low nitrite dose, increased amounts of sodium ascorbate resulted in less lipid oxidation. Decreasing the nitrite dose to 80 or 40 mg/kg had no distinct effect on protein oxidation.

In chapter 3 the effect of reduced nitrite liver pâtés, using a pre-converted plant extract as source of nitrite, on the oxidative stability during chilled display was investigated. Lowering the ingoing nitrite levels to 90, 45 and 20 mg/kg did not affect the colour formation and colour stability, compared to a positive control containing 120 mg/kg sodium nitrite. Inconsistent results for the treatment effects on the protein carbonyls content of the liver pâtés were found. After nine days of illuminated chilled display, lowest amounts of hexanal (a lipid oxidation product) were formed in the treatments with 45 and 90 mg/kg ingoing nitrite, which suggests that the antioxidant effect of sodium nitrite was partly replaced by other compounds present in the extract.

In chapter 4 the role of sodium nitrite on protein oxidation has been investigated from a more mechanistic point of view and a marker for protein nitration, 3-nitrotyrosine, was introduced in meat. Two meat models were investigated: isolated pig myofibrilar protein suspensions and raw pork patties. In the isolated pig myofibrillar protein suspensions, sodium nitrite initially (30 min after inducing oxidation with pro-oxidants) showed a pro-oxidative effect for protein carbonyl formation, but during the incubation at 37°C, no effect of sodium nitrite was observed. On the contrary, sodium nitrite tended to act as an antioxidant against protein carbonyl formation in raw pork muscles. However, this effect was observed in one batch of patties, while in the other batch no effect of sodium nitrite was found. The marker 3-nitrotyrosine was retrieved in all samples, but no clear effect of sodium nitrite was observed during illuminated chilled display.

For many years already, but with little success, researchers are trying to reproduce the characteristic pink colour without the use of nitrite. Consequently, one could question whether it

is feasible to add very small amounts of nitrite to produce the cured colour and improve the decreased colour stability using other active compounds. Nitrite is a powerful antioxidant against lipid oxidation, but a dog rose extract, pre-converted extract and sodium ascorbate are potential substitutes. Less clear is the effect of lowering nitrite on protein oxidation and whether nitrite has an effect on protein oxidation at all. Without ignoring the fact that meat and especially cured meat products contribute to an increased risk of cancer development, we should revise our long-standing view that nitrate and nitrite are only harmful substances in our diet, as therapeutic benefits of nitrite against diseases associated with nitric oxide insufficiency were recently reported.

Chapter 5 describes an experiment in which different n-3 PUFA sources were added to pig feed and its effect on the fatty acid profile of fresh loin, dry fermented sausage and long ripened dry cured ham was investigated. Crossbred pigs were given an experimental diet supplemented with 0.6 g/100g linseed oil (LIN), 0.8 g/100g fish oil (FISH) or dried microalgae (ALG). Three ALG groups were considered: ALG LOW, ALG MEDIUM and ALG HIGH with respectively 0.3, 0.6 and 1.2 g dried microalgae per 100 g feed. In the control group (SOY) soybean oil was added to the diet. Similar results were found for the different meat products investigated. Compared to all other groups, significantly higher C18:3n-3 (ALA) proportions in the LIN group and higher proportions of C20:5n-3 (EPA) and C22:6n-3 (DHA) in the FISH and ALG groups were found. The EPA proportions of the meat products from the LIN group were slightly but significantly higher compared to the SOY group. It was calculated that the daily recommended intake of EPA and DHA can be increased considerably with these n-3 polyunsaturated fatty acids enriched products.

The experiment in chapter 6 was performed to investigate the sensory quality of the n-3 fatty acids enriched meat products described in chapter 5. Colour and colour stability, lipid oxidation and consumers' acceptability was assessed. Manufacturing processes such as fermentation and

long term dry curing affected the oxidative stability of n-3 PUFA enriched meat products as colour and lipid oxidation parameters were inferior in the dry fermented sausages and dry cured ham when comparing to the conventional counterparts. The n-3 PUFA enriched loin did not differ from the control group. Problems of fishy and rancid off-flavours in the ALG HIGH groups were reported by the consumer panelists in the dry fermented sausages, but not in the dry cured hams.

In chapter 7, the effect of supplementing supra-nutritional levels of α-tocopherol (95, 175 and 400 mg/kg feed) on the oxidative stability of n-3 PUFA enriched pork products was described. The susceptibility to lipid oxidation was not improved in the loin and dry fermented sausages and the initial colour values of the loin were not affected. Some modest colour improvements in the dry fermented sausages indicate a slight positive effect of the α-tocopherol supplementation. Colour stability of defrosted loins and dry fermented sausages was not affected by the investigated dietary α-tocopherol levels.

In chapter 8 the effect of consuming n-3 PUFA enriched meat on the health status was assessed. Health biomarkers were evaluated in rabbits as a model for humans. A diet based on the human consumption pattern was formulated and contained pork enriched with n-3 fatty acids originating from linseed oil (ALA) or fish oil (DHA+EPA) fed pigs. The fatty acid profile of different tissues and blood reflected the fatty acid composition of the diet. The oxidative status of the rabbits was affected by the dietary cholestorol which was added to induce atherosclerosis, but not by the fatty acid profile of the diet. During the experiment, the total cholesterol/HDL-cholesterol ratio decreased and the total cholesterol/LDL-cholesterol ratio increased in the fish oil pork group, while for the linseed oil pork group the total cholesterol/HDL-cholesterol ratio increased and no effect was measured for the total cholesterol/LDL-cholesterol ratio. Atherosclerosis developed during the experiment, but no difference between the two feeding groups was found.

Similar to humans, the conversion of ALA to EPA in pigs is low, pointing out the importance of using adequate sources for EPA and DHA in the feed to achieve improved products beneficial to health. Microalgae are a good alternative to replace fish oil as source of EPA and DHA in pig feed, without pressuring the marine ecosystem. As the sensory quality of enriched products greatly depends on the absolute EPA and DHA content, the focus should not be on strongly increasing the EPA and DHA content of one or a few food products, but on enriching a number of widely consumed food products with moderate amounts of EPA and DHA. As meat is one of the main sources of long chain n-3 PUFA, even moderate improvements of its fatty acid composition is valuable. In this view it is of interest to enrich meat through animal feeding, as the enriched tissues are entering the food chain through the production of various n-3 PUFA enriched meat products.

SAMENVATTING

Epidemiologisch onderzoek heeft uitgewezen dat slechte voedingsgewoonten het risico op chronische aandoeningen verhogen. Naast initiatieven om mensen te sensibiliseren tot een gezonder eetpatroon, is het optimaliseren van de samenstelling van veel gebruikte voedingsmiddelen een andere strategie om tot een eetpatroon te komen dat beter aanleunt bij de voedingsaanbevelingen.

Er wordt o.a. aanbevolen om meer producten te consumeren die rijk zijn aan EPA (C20:5n-3) en DHA (C22:6n-3), omdat deze meervoudige onverzadigde vetzuren de kans op hart- en vaatziekten verlagen. Ook wordt er aanbevolen om de nitrietinname te verminderen wegens mogelijks carcinogeen. Nitriet is een kleurstabiliserend additief met antibacteriële, smaakvormende en antioxidant eigenschappen. Dit proefschrift heeft zich toegespitst op vlees en vleesproducten en heeft twee specifieke onderzoeksdoelstellingen: de effecten op de sensorische kwaliteit en oxidatieve stabiliteit (vet-, pigment- en eiwitoxidatie) nagaan van het verlagen van het nitrietgehalte in gekookte vleeswaren (hoofdstukken 1-4) en van de aanrijking van vlees(producten) met n-3 vetzuren via het varkensvoeder (hoofdstukken 5-8).

In hoofdstuk 1 werd onderzocht of een extract van hondsroos (*Rosa canina* L.) de rol van nitriet kan vervangen in nitriet-vrije frankfurters. Hondsroos is een bron van ascorbinezuur, polyfenolen en nitraat. Het extract werd in een dosis van 5 of 30 g/kg toegevoegd aan frankfurters. Deze frankfurters bevatten geen natriumnitriet of natriumascorbaat en werden vergeleken met een positieve controle (100 mg/kg natriumnitriet en 500 mg/kg natriumascorbaat). De experimentele frankfurters vertoonden dezelfde mate van vetoxidatie als de positieve controle, terwijl de eiwitoxidatie hoger was. Aangezien de kleureigenschappen van de experimentele frankfurters niet hetzelfde waren als die van de positieve controle, werd besloten dat hondsroos een goede

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natuurlijke antioxidant is voor toepassing in frankfurters, maar niet gebruikt kan worden als volwaardige vervanger voor nitriet.

In hoofdstuk 2 werd nagegaan of een verhoogde dosis natriumascorbaat (750 en 1000 mg/kg i.p.v. 500 mg/kg) de rol van nitriet kan opvangen in leverpâté, wanneer 40 of 80 mg/kg natriumnitriet wordt toegediend i.p.v. 120 mg/kg. Het nitrietgehalte verlagen naar 80 mg/kg had geen negatieve impact op de kleurvorming, kleurstabiliteit of vetoxidatie en het ascorbaatgehalte verhogen had geen bijkomend positief effect. Wanneer het nitrietgehalte verder verlaagd werd naar 40 mg/kg was de kleur hetzelfde als de pâtés met 120 mg/kg nitriet, maar de kleur- en vetstabiliteit waren minder goed. Wanneer bij deze nitrietdosis 750 of 1000 mg/kg ascorbaat werd toegevoegd, trad er minder vetoxidatie op vergeleken met 500 mg/kg. De verlaagde nitrietgehaltes of verhoogde ascorbaatgehaltes hadden geen effect op eiwitoxidatie. In conclusie kan men stellen dat het verhogen van ascorbaat nuttig is t.o.v. vetoxidatie, maar enkel wanneer verlaagd wordt naar 40 mg/kg nitriet.

In hoofdstuk 3 werd nitriet afkomstig van een natuurlijk extract gebruikt om leverpâté's met een verlaagd nitrietgehalte (20, 45 en 90 mg/kg) te produceren. De kleur en kleurstabiliteit werden niet beïnvloed door de verlaagde nitrietgehaltes, terwijl onverwacht minder vetoxidatie optrad, vergeleken met een positieve controle (120 mg/kg natriumnitriet). Dit wijst er op dat het plantenextract naast nitriet nog andere antioxidant componenten bevatte. Tijdens de blootstelling van deze producten werden eiwitten geoxideerd, maar het was onduidelijk of nitriet hier een invloed op had.

In hoofdstuk 4 werd meer specifiek gekeken naar de rol van nitriet m.b.t. eiwitoxidatie en werd 3-nitrotyrosine geïntroduceerd als merker voor eiwitnitratie in vlees. Helaas, opnieuw kon geen eenduidig besluit genomen worden of nitriet een pro- of antioxidant effect heeft op eiwitoxidatie, aangezien een pro-oxidant effect in geïsoleerde myofibrillen en een antioxidant effect in rauwe varkensburgers werd waargenomen. De merker 3-nitrotyrosine werd in alle stalen teruggevonden,

maar een duidelijk effect van nitriet op 3-nitrotyrosine tijdens blootstelling aan licht en lucht werd niet gevonden.

Kortom, het is mogelijk om het nitrietgehalte in vleeswaren sterk te verlagen zonder dat de kleur en kleurstabiliteit drastisch beïnvloed worden en de vetoxidatie kan opgevangen worden door andere (natuurlijke) componenten. Het is niet duidelijk of eiwitoxidatie beïnvloed wordt bij lagere nitrietgehaltes. Anderzijds is het volledig weglaten van nitriet moeilijk, aangezien het niet mogelijk is om de gewenste roze kleur te reproduceren. Bovendien kan de vraag gesteld worden of het nodig is om nitriet volledig te bannen uit ons dieet, aangezien stikstofmonoxide, o.a. afkomstig van nitriet, ook een belangrijke regulerende functie heeft in het menselijk lichaam.

Hoofdstuk 5 beschrijft een experiment waarbij getracht werd het vetzuurprofiel van vers varkensvlees, gedroogde ham en salami te wijzigen door varkens een voeder aangerijkt met lijnzaadolie (0,6 g/100g), visolie (0,8 g/100g) of algen (0,3; 0, 6 of 1,2 g/100g) te geven. De controlegroep kreeg een standaard voeder op basis van soja olie. Voor de drie vleesproducten konden gelijkaardige besluiten getrokken worden: vergeleken met de controle zorgde de lijnzaadolie in het voeder voor hogere ALA (C18:3n-3) en - in mindere mate - EPA concentraties, terwijl toediening van visolie of algen resulteerde in verhoogde EPA en DHA concentraties in de vleesproducten. Aan de hand van deze data werd berekend dat de consumptie van een portie aangerijkte vleesproducten duidelijk bijdraagt tot een verhoogde inname van EPA en DHA.

In hoofdstuk 6 werd vervolgens onderzocht welk effect de verhoogde concentratie onverzadigde vetzuren heeft op de kleur, kleurstabiliteit, vetoxidatie en sensorische kwaliteit van de aangerijkte producten. De kwaliteit van het verse vlees was niet veranderd door de verhoogde concentraties aan n-3 vetzuren, maar de kleur en oxidatieve stabiliteit van de gedroogde hammen en salami's waren wel negatief beïnvloed. Bovendien werden de salami's afkomstig van de

varkens gevoederd met de hoogste dosis algen afgekeurd door het consumentenpanel, wegens de aanwezigheid van onaangename ranzige geuren en smaken.

Om de oxidatieve stabiliteit van salami's die aangerijkt werden met n-3 vetzuren te verbeteren, werd een experiment uitgevoerd waarbij visolie en hoge dosissen α-tocoferol (95, 175 and 400 mg/kg) toegevoegd werden aan varkensvoeder (Hoofdstuk 7). Naarmate meer α-tocoferol aanwezig was in het voeder, werden ook hogere concentraties α-tocoferol teruggevonden in het vet en vlees, maar dit had geen effect op de oxidatieve stabiliteit van het verse vlees of de salami.

In hoofdstuk 8 werd onderzocht of de consumptie van vlees aangerijkt met n-3 vetzuren een effect heeft op de gezondheid. Hiervoor werd een konijn als model voor de mens gebruikt. De dieren kregen een experimenteel voeder toegediend dat gebaseerd is op het westers dieet en waarvan het varkensvlees en rugspek afkomstig was van varkens die gevoederd werden met ofwel lijnzaad ofwel visolie. Er werd 1 % cholesterol toegevoegd om atherosclerose te induceren. Het vetzuurprofiel van het hart, de aorta, het plasma en de rode bloedcellen vertoonden allen dezelfde trend: hogere EPA en DHA gehaltes in de visoliegroep vergeleken met de lijnzaadgroep en voor de andere vetzuren weinig verschillen tussen de twee groepen. Alle konijnen vertoonden atherosclerose, maar er werden geen significante verschillen tussen de groepen waargenomen. Ook wat de endogene antioxidatieve enzymen betreft, werden geen significante verschillen gevonden. Tijdens het experiment daalde de verhouding totaal cholesterol:HDL-cholesterol en steeg de verhouding totaal cholesterol:LDL-cholesterol in de visoliegroep, terwijl deze verhouding respectievelijk steeg en hetzelfde bleef in de lijnzaadgroep.

Net zoals bij de mens is de omzetting van ALA naar EPA in het varken laag en is het belangrijk om de juiste n-3 vetzuurbronnen te gebruiken wanneer men vleesproducten wil maken met verhoogde EPA en DHA concentraties. Algen zijn een goed alternatief voor visolie omdat ze ook een bron zijn van DHA en EPA, en ze geen druk uitoefenen op het marien ecosysteem. Aangezien de sensorische kwaliteit sterk afhangt van de concentratie aan EPA en DHA, is het

niet aangewezen om voedingswaren aan te rijken met zeer hoge concentraties, maar dient de aandacht te gaan naar het aanrijken van meerdere veelvuldig gebruikte voedingsmiddelen met een matige dosis van deze vetzuren. Aangezien vlees en vleeswaren veel geconsumeerd worden, is het nuttig om deze ook aan te rijken met matige hoeveelheden EPA en DHA. In deze context is het interessant om dit via het diervoeder te doen, omdat op die manier de samenstelling van alle spieren en weefsels gestuurd wordt en hierdoor zowel vers vlees als een breed gamma van aangerijkte vleesproducten in de voedselketen terecht komt.

Adarme-Vega, T. C., Lim, D. K., Timmins, M., Vernen, F., Li, Y., & Schenk, P. M. (2012). Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. *Microbial Cell Factories, 11,* 96-106.

Aebi, H. & Bergmeyer, H. (1974). Catalase. *In: Methods of Enzymatic Analysis,* 673-677. Academic Press, New York.

Ahn, D. U., Sell, J. L., Jo, C., Chen, X., Wu, C., & Lee, J. I. (1998). Effects of dietary vitamin E supplementation on lipid oxidation and volatiles content of irradiated, cooked turkey meat patties with different packaging. *Poultry Science, 77,* 912-920.

Akagawa, M., Sasaki, D., Ishii, Y., Kurota, Y., Yotsu-Yamashita, M., Uchida, K. et al. (2006). New method for the quantitative determination of major protein carbonyls, α-aminoadipic and γ-glutamic semialdehydes: investigation of the formation mechanism and chemical nature in vitro and in vivo. *Chemical Research in Toxicology, 19,* 1059-1065.

Alipour, M., Mohammadi, M., Zarghami, N., & Ahmadiasl, N. (2006). Influence of chronic exercise on red cell antioxidant defense, plasma malondialdehyde and total antioxidant capacity in hypercholesterolemic rabbits. *Journal of Sports Science & Medicine, 5,* 682-691.

American Institute for Cancer Research (2007). *World cancer research report* Washington, DC: American Institute for Cancer Research.

Amici, A., Levine, R. L., Tsai, L., & Stadtman, E. R. (1989). Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *Journal of Biological Chemistry, 264,* 3341-3346.

AOAC (2000). *Offical Methods of Analysis*. (17 ed.) Gaithersburgh, Maryland.

AOCS Lipid Library (2014). *http://lipidlibrary.aocs.org* Accessed 20/03/2014.

Aranceta, J. & Pérez-Rodrigo, C. (2012). Recommended dietary reference intakes, nutritional goals and dietary guidelines for fat and fatty acids: a systematic review. *British Journal of Nutrition, 107,* S8-S22.

Arendt, B., Skibsted, L. H., & Andersen, H. J. (1997). Antioxidative activity of nitrite in metmyoglobin induced lipid peroxidation. *Zeitung für Lebensmittel-Untersuchung und Forschung, 204,* 7-12.

Armenteros, M., Heinonen, M., Ollilainen, V., Toldrá, F., & Estévez, M. (2009). Analysis of protein carbonyls in meat products by using the DNPH-method, fluorescence spectroscopy and liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS). *Meat Science, 83,* 104-112.

Association Francaise de Normalisation (2000). Norme V09-500. Directives generales pour la realisation d'epreuves hedoniques en laboratoire d'evaluation sensorielle ou en salle en conditions controlees impliquant des consommateurs. In *AFNOR, Recueil de normes. Analyse sensorielle. Association Francaise de Normalisation,* 227-256. Paris, France.

Aumaitre, A., Aulrich, K., Chesson, A., Flachowsky, G., & Piva, G. (2002). New feeds from genetically modified plants: substantial equivalence, nutritional equivalence, digestibility, and safety for animals and the food chain. *Livestock Production Science, 74,* 223-238.

Batifoulier, F., Mercier, Y., Gatellier, P., & Renerre, M. (2002). Influence of vitamin E on lipid and protein oxidation induced by H_2O_2-activated MetMb in microsomal membranes from turkey muscle. *Meat Science, 61,* 389-395.

Benzie, I. 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.

Blank, C., Neumann, M. A., Makrides, M., & Gibson, R. A. (2002). Optimizing DHA levels in piglets by lowering the linoleic acid to α-linolenic acid ratio. *Journal of Lipid Research, 43,* 1537-1543.

Botsoglou, E., Govaris, A., Ambrosiadis, I., & Fletouris, D. (2012). Lipid and protein oxidation of α-linolenic acid-enriched pork during refrigerated storage as influenced by diet supplementation with olive leaves (*Olea europea* L.) or α-tocopheryl acetate. *Meat Science, 92,* 525-532.

Botsoglou, E., Govaris, A., Pexara, A., Ambrosiadis, I., & Fletouris, D. (2014). Effect of dietary olive leaves (*Olea europaea* L.) on lipid and protein oxidation of refrigerated stored n-3 enriched pork. *International Journal of Food Science & Technology, 49,* 42-50.

Bou, R., Grimpa, S., Guardiola, F., Barroeta, A. C., & Codony, R. (2006). Effects of various fat sources, α−tocopheryl acetate, and ascorbic acid supplements on fatty acid composition and α-tocopherol content in raw and vacuum-packed, cooked dark chicken meat. *Poultry Science, 85,* 1472-1481.

Bourne, M. C. (1982). *Food Texture and Viscosity.* (1 ed.) New York: Academic Press, Inc.

Bramley, P. M., Elmadfa, I., Kafatos, A., Kelly, F., Manios, Y., Roxborough, H. et al. (2000). Vitamin E. *Journal of the Science of Food and Agriculture, 80,* 913-938.

Brannan, R. G., Connolly, B. J., & Decker, E. A. (2001). Peroxynitrite: a potential initiator of lipid oxidation in food. *Trends in Food Science & Technology, 12,* 164-173.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56,* 317-333.

Brenna, J. T. (2002). Efficiency of conversion of α-linolenic acid to long chain n-3 fatty acids in man. *Current Opinion in Clinical Nutrition & Metabolic Care, 5,* 127-132.

Brown, C. L., Hedrick, H. B., & Bailey, M. E. (1974). Characteristics of cured ham as influenced by levels of sodium nitrite and sodium ascorbate. *Journal of Food Science, 39,* 977-979.

Brunner, E. J., Jones, P. J., Friel, S., & Bartley, M. (2009). Fish, human health and marine ecosystem health: policies in collision. *International Journal of Epidemiology, 38,* 93-100.

Bunnell, R. H., Keating, J., Quaresimo, A., & Parman, G. K. (1965). Alpha-tocopherol content of foods. *The American Journal of Clinical Nutrition, 17,* 1-10.

Burdge, G. (2004). α-Linolenic acid metabolism in men and women: nutritional and biological implications. *Current Opinion in Clinical Nutrition & Metabolic Care, 7,* 137-144.

Cakilcioglu, U. & Khatun, S. (2011). Nitrate, moisture and ash contents of edible wild plants. *Journal of Cell & Plant Science, 2,* 1-5.

Cando, D., Morcuende, D., Utrera, M., & Estévez, M. (2014). Phenolic-rich extracts from Willowherb (*Epilobium hirsutum* L.) inhibit lipid oxidation but accelerate protein carbonylation and discoloration of beef patties. *European Food Research and Technology, 5,* 741-751.

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

Chizzolini, R., Novelli, E., & Zanardi, E. (1998). Oxidation in traditional Mediterranean meat products. *Meat Science, 49,* S87-S99.

Claeys, E., Vossen, E., & De Smet, S. (2014). Determination of alpha-tocopherol by reversed phase HPLC in animal-derived foods and in feed without saponification. *under review*.

Coates, A. M., Sioutis, S., Buckley, J. D., & Howe, P. R. (2009). Regular consumption of n-3 fatty acid-enriched pork modifies cardiovascular risk factors. *British Journal of Nutrition, 101,* 592-597.

Connolly, B. J. & Decker, E. A. (2004). Peroxynitrite induced discoloration of muscle foods. *Meat Science, 66,* 499-505.

Coutron-Gambotti, C. & Gandemer, G. (1999). Lipolysis and oxidation in subcutaneous adipose tissue during dry-cured ham processing. *Food Chemistry, 64,* 95-101.

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.

Deckelbaum, R. J., Leaf, A., Mozaffarian, D., Jacobson, T. A., Harris, W. S., & Akabas, S. R. (2008). Conclusions and recommendations from the symposium, Beyond Cholesterol: prevention and treatment of coronary heart disease with n−3 fatty acids. *The American Journal of Clinical Nutrition, 87,* 2010S-2012S.

Decker, E. A. (1998). Strategies for manipulating the prooxidative/antioxidative balance of foods to maximize oxidative stability. *Trends in Food Science & Technology, 9,* 241-248.

Demeyer, D., Honikel, K., & De Smet, S. (2008). The world cancer research fund report 2007: A challenge for the meat processing industry. *Meat Science, 80,* 953-959.

Demir, F. & Ízcan, M. (2001). Chemical and technological properties of rose (*Rosa canina* L.) fruits grown wild in Turkey. *Journal of Food Engineering, 47,* 333-336.

Desai, I. D. (1984). Vitamin E analysis methods for animal tissues. *Methods in Enzymology, 105,* 138-147.

Devriese, S., Huybrechts, I., Moreau, M., & Van Oyen, H. (2006). De Belgische Voedselconsumptiepeiling 1-2004. *Wetenschappelijk Instituut Volksgezondheid.*

Dineen, N., Kerry, J. P., Buckley, D. J., Morrissey, P. A., Arendt, E. K., & Lynch, P. B. (2001). Effect of dietary α−tocopheryl acetate supplementation on the shelf-life stability of reduced nitrite cooked ham products. *International Journal of Food Science & Technology, 36,* 631-639.

Dodson, K. Y., Young, E. R., & Soliman, A. G. M. (1992). Determination of total vitamin C in various food matrices by liquid-chromatography and fluorescence detection. *Journal of the Association of Official Analytical Chemists International, 75,* 887-891.

Dong, Q., Tu, K., Guo, L., Yang, J. L., Wang, HI., & Chen, Y. Y. (2007). The effect of sodium nitrite on the textural properties of cooked sausage during cold storage. *Journal of Texture Studies, 38,* 537-554.

Doolaege, E. H., Vossen, E., Raes, K., De Meulenaer, B., Verhé, R., Paelinck, H. et al. (2012). Effect of rosemary extract dose on lipid oxidation, colour stability and antioxidant concentrations, in reduced nitrite liver pâtés. *Meat Science, 90,* 925-931.

Dufresne, C. & Farnworth, E. (2000). Tea, Kombucha, and health: a review. *Food Research International, 33,* 409-421.

Egea, I., Sánchez-Bel, P., Romojaro, F., & Pretel, M. (2010). Six edible wild fruits as potential antioxidant additives or nutritional supplements. *Plant Foods for Human Nutrition, 65,* 121-129.

Elmore, J. S., Cooper, S. L., Enser, M., Mottram, D. S., Sinclair, L. A., Wilkinson, R. G. et al. (2005). Dietary manipulation of fatty acid composition in lamb meat and its effect on the volatile aroma compounds of grilled lamb. *Meat Science, 69,* 233-242.

Elmore, J. S., Mottram, D. S., Enser, M., & Wood, J. D. (1999). Effect of the polyunsaturated fatty acid composition of beef muscle on the profile of aroma volatiles. *Journal of Agricultural and Food Chemistry, 47,* 1619-1625.

Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science, 89,* 259-279.

Estévez, M. & Cava, R. (2006a). Effectiveness of rosemary essential oil as an inhibitor of lipid and protein oxidation: Contradictory effects in different types of frankfurters. *Meat Science, 72,* 348-355.

Estévez, M. & Heinonen, M. (2010). Effect of phenolic compounds on the formation of alpha-aminoadipic and gamma-glutamic semialdehydes from myofibrillar proteins oxidized by copper, iron, and myoglobin. *Journal of Agricultural and Food Chemistry, 58,* 4448-4455.

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., & Heinonen, M. (2008). Oxidation of skeletal muscle myofibrillar proteins in oil-in-water emulsions: interaction with lipids and effect of selected phenolic compounds. *Journal of Agricultural and Food Chemistry, 56,* 10933-10940.

Estévez, M., Morcuende, D., Ventanas, S., & Cava, R. (2003). Analysis of volatiles in meat from Iberian pigs and lean pigs after refrigeration and cooking by using SPME-GC-MS. *Journal of Agricultural and Food Chemistry, 51,* 3429-3435.

Estévez, M., Ollilainen, V., & Heinonen, M. (2009). Analysis of protein oxidation markers alpha-aminoadipic and gamma-glutamic semialdehydes in food proteins using liquid chromatography (LC)-electrospray ionization (ESI)-multistage tandem mass spectrometry (MS). *Journal of Agricultural and Food Chemistry, 57,* 3901-3910.

Estévez, M., Ramirez, R., Ventanas, S., & Cava, R. (2007a). Sage and rosemary essential oils versus BHT for the inhibition of lipid oxidative reactions in liver pâté. *LWT-Food Science and Technology, 40,* 58-65.

Estévez, M., Ventanas, S., Heinonen, M., & Puolanne, E. (2011). Protein carbonylation and water-holding capacity of pork subjected to frozen Storage: effect of muscle type, premincing, and packaging. *Journal of Agricultural and Food Chemistry, 59,* 5435-5443.

Estévez, M., Ventanas, S., Ramirez, R., & Cava, R. (2005a). Influence of the addition of rosemary essential oil on the volatiles pattern of porcine frankfurters. *Journal of Agricultural and Food Chemistry, 53,* 8317-8324.

Estévez, M., Ventanas, S., & Cava, R. (2005b). Physicochemical properties and oxidative stability of liver pâté as affected by fat content. *Food Chemistry, 92,* 449-457.

Estévez, M., Ventanas, S., & Cava, R. (2006b). Effect of natural and synthetic antioxidants on protein oxidation and colour and texture changes in refrigerated stored porcine liver pâté. *Meat Science, 74,* 396-403.

Estévez, M., Ventanas, S., & Cava, R. (2007b). Oxidation of lipids and proteins in frankfurters with different fatty acid compositions and tocopherol and phenolic contents. *Food Chemistry, 100,* 55-63.

European Commission (2010). Regulation (EU) No 116/2010 of 9 February 2010 amending Regulation (EC) No 1924/2006 of the European Parliament and of the Council with regard to the list of nutrition claims. *Official Journal of the European Union L, 37,* 16-18.

European Food Safety Authority (2003). Opinion of the scientific panel on biological hazards on the request from the commission related to the effects of nitrites/nitrates on the microbiological safety of meat products. *EFSA Journal, 14,* 1-13.

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science*, 86, 86-94.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2008). Lyon, France: International agency for research on cancer; 2010. *Cancer Incidence and Mortality Worldwide: IARC CancerBase*.

Fernando, L. N., Berg, E. P., & Grün, I. U. (2003). Quantitation of hexanal by automated SPME for studying dietary influences on the oxidation of pork. *Journal of Food Composition and Analysis, 16,* 179-188.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry, 226,* 497-509.

Fontana, M., Blarzino, C., & Pecci, L. (2012). Formation of 3-nitrotyrosine by riboflavin photosensitized oxidation of tyrosine in the presence of nitrite. *Amino Acids, 42,* 1857-1865.

Fox Jr, J. B. (1987). The pigments of meat. In J. F. Price & B. Schweigert (Eds.), *The science of meat and meat products* (3rd ed.), 1-148. Westport, CT: Food and Nutrition Press, Inc.

Frankel, E. N. (1984). Lipid oxidation: mechanisms, products and biological significance. *Journal of the American Oil Chemists' Society, 61,* 1908-1917.

Fujimaki, M., Emi, M., & Okitani, A. (1975). Fate of nitrite in meat-curing model systems composed of myoglobin, nitrite and ascorbate. *Agricultural and Biological Chemistry, 39.* 371-377.

Ganhão, R., Estévez, M., Kylli, P., Heinonen, M., & Morcuende, D. (2010a). Characterization of selected wild Mediterranean fruits and comparative efficacy as inhibitors of oxidative reactions in emulsified raw pork burger patties. *Journal of Agricultural and Food Chemistry, 58,* 8854-8861.

Ganhão, R., Morcuende, D., & Estévez, M. (2010b). Protein oxidation in emulsified cooked burger patties with added fruit extracts: Influence on colour and texture deterioration during chill storage. *Meat Science, 85,* 402-409.

Ganhão, R., Morcuende, D., & Estévez, M. (2010c). Tryptophan depletion and formation of alpha-aminoadipic and gamma-glutamic semialdehydes in porcine burger patties with added phenolic-rich fruit extracts. *Journal of Agricultural and Food Chemistry, 58,* 3541-3548.

Garcia-Esteban, M., Ansorena, D., Astiasarán, I., & Ruiz, J. (2004). Study of the effect of different fiber coatings and extraction conditions on dry cured ham volatile compounds extracted by solid-phase microextraction (SPME). *Talanta, 64,* 458-466.

García-Íñiguez de Ciriano, M., García-Herreros, C., Larequi, E., Valencia, I., Ansorena, D., & Astiasarán, I. (2009). Use of natural antioxidants from lyophilized water extracts of *Borago officinalis* in dry fermented sausages enriched in ω-3 PUFA. *Meat Science, 83,* 271-277.

García-Íñiguez de Ciriano, M., Larequi, E., Rehecho, S., Calvo, M. I., Cavero, R. Y., Navarro-Blasco, Ì. et al. (2010). Selenium, iodine, ω-3 PUFA and natural antioxidant from *Melissa officinalis* L.: A combination of components from healthier dry fermented sausages formulation. *Meat Science, 85,* 274-279.

Garg, M. L., Blake, R. J., Clayton, E., Munro, I. A., Donald-Wicks, L., Singh, H. et al. (2007). Consumption of an n-3 polyunsaturated fatty acid-enriched dip modulates plasma lipid profile in subjects with diabetes type II. *European journal of clinical nutrition, 61,* 1312-1317.

Gatellier, P., Kondjoyan, A., Portanguen, S., & Santé-Lhoutellier, V. (2010). Effect of cooking on protein oxidation in n-3 polyunsaturated fatty acids enriched beef. Implication on nutritional quality. *Meat Science, 85,* 645-650.

Gebert, S., Eichenberger, B., Pfirter, H. P., & Wenk, C. (2006). Influence of different dietary vitamin C levels on vitamin E and C content and oxidative stability in various tissues and stored m. *longissimus dorsi* of growing pigs. *Meat Science, 73,* 362-367.

Gibson, A. M., Roberts, T. A., & Robinson, A. (1984). Factors controlling the growth of Clostridium botulinum types A and B in pasteurized cured meats VI. Nitrite monitoring during storage of pasteurized pork slurries. *International Journal of Food Science & Technology, 19,* 29-44.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry, 177,* 751-766.

Gray, J. I. (1978). Measurement of lipid oxidation: a review. *Journal of the American Oil Chemists' Society, 55,* 539-546.

Gray, J. I., Gomaa, E. A., & Buckley, D. J. (1996). Oxidative quality and shelf life of meats. *Meat Science, 43,* 111-123.

Grunert, K. G. (1997). What's in a steak? A cross-cultural study on the quality perception of beef. *Food Quality and Preference, 8,* 157-174.

Haak, L., De Smet, S., Fremaut, D., Van Walleghem, K., & Raes, K. (2008). Fatty acid profile and oxidative stability of pork as influenced by duration and time of dietary linseed or fish oil supplementation. *Journal of Animal Science, 86,* 1418-1425.

Haak, L., Raes, K., & De Smet, S. (2009). Effect of plant phenolics, tocopherol and ascorbic acid on oxidative stability of pork patties. *Journal of the Science of Food and Agriculture, 89,* 1360-1365.

Heaton, K. M., Cornforth, D. P., Moiseev, I. V., Egbert, W. R., & Carpenter, C. E. (2000). Minimum sodium nitrite levels for pinking of various cooked meats as related to use of direct or indirect-dried soy isolates in poultry rolls. *Meat Science, 55,* 321-329.

Heinonen, M. (2007). Antioxidant activity and antimicrobial effect of berry phenolics-a Finnish perspective. *Molecular Nutrition & Food Research, 51,* 684-691.

Hernández, P., Zomeno, L., Ariño, B., & Blasco, A. (2004). Antioxidant, lipolytic and proteolytic enzyme activities in pork meat from different genotypes. *Meat Science, 66,* 525-529.

Holownia, K., Chinnan, M. S., & Reynolds, A. E. (2011). Pink color defect in poultry white meat as affected by endogenous conditions. *Journal of Food Science, 68,* 742-747.

Honikel, K. O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science, 78,* 68-76.

Houdebine, L. M. (2014). Impacts of genetically modified animals on the ecosystem and human activities. *Global Bioethics, 25,* 3-18.

Howe, P., Meyer, B., Record, S., & Baghurst, K. (2006). Dietary intake of long-chain n-3 polyunsaturated fatty acids: contribution of meat sources. *Nutrition, 22,* 47-53.

Hoz, L., D'Arrigo, M., Cambero, I., & Ordóñez, J. A. (2004). Development of an n-3 fatty acid and α-tocopherol enriched dry fermented sausage. *Meat Science, 67,* 485-495.

Hsu, H. C., Lee, Y. T., & Chen, M. F. (2001). Effects of fish oil and vitamin E on the antioxidant defense system in diet-induced hypercholesterolemic rabbits. *Prostaglandins & other Lipid Mediators, 66,* 99-108.

Hussein, N., Ah-Sing, E., Wilkinson, P., Leach, C., Griffin, B. A., & Millward, D. J. (2005). Long-chain conversion of [13C] linoleic acid and α-linolenic acid in response to marked changes in their dietary intake in men. *Journal of Lipid Research, 46,* 269-280.

Igene, J. O., Yamauchi, K., Pearson, A. M., Gray, J. I., & Aust, S. D. (1985). Mechanisms by which nitrite inhibits the development of warmed-over flavour (WOF) in cured meat. *Food Chemistry, 18,* 1-18.

Iglesias, J. & Medina, I. (2008). Solid-phase microextraction method for the determination of volatile compounds associated to oxidation of fish muscle. *Journal of Chromatography A, 1192,* 9-16.

Izumi, K., Cassens, R. G., & Greaser, M. L. (1989). Reaction of nitrite with ascorbic acid and its significant role in nitrite-cured food. *Meat Science, 26,* 141-153.

Jayabalan, R., Subathradevi, P., Marimuthu, S., Sathishkumar, M., & Swaminathan, K. (2008). Changes in free-radical scavenging ability of kombucha tea during fermentation. *Food Chemistry*, 109, 227-234.

Jensen, C., Lauridsen, C., & Bertelsen, G. (1998). Dietary vitamin E: quality and storage stability of pork and poultry. *Trends in Food Science & Technology, 9,* 62-72.

Johnson, G. H. & Fritsche, K. (2012). Effect of dietary linoleic acid on markers of inflammation in healthy persons: A systematic review of randomized controlled trials. *Journal of the Academy of Nutrition and Dietetics, 112,* 1029-1041.

Jongberg, S., Torngren, M. A., Gunvig, A., Skibsted, L. H., & Lund, M. N. (2013). Effect of green tea or rosemary extract on protein oxidation in Bologna type sausages prepared from oxidatively stressed pork. *Meat Science, 93,* 538-546.

Juárez, M., Dugan, M. E. R., Larsen, I. L., Thacker, R., Rolland, D. C., & Aalhus, J. L. (2011). Oxidative stability in grinds from omega-3 enhanced pork. *Canadian Journal of Animal Science, 91,* 623-634.

Kaack, K., Lærke, H. N., & Meyer, A. S. (2006). Liver paté enriched with dietary fibre extracted from potato fibre as fat substitutes. *European Food Research and Technology, 223,* 267-272.

Kähkönen, M. P. & Heinonen, M. (2003). Antioxidant activity of anthocyanins and their aglycons. *Journal of Agricultural and Food Chemistry, 51,* 628-633.

Kang, M. J., Shin, M. S., Park, J. N., & Lee, S. S. (2005). The effects of polyunsaturated: saturated fatty acids ratios and peroxidisability index values of dietary fats on serum lipid profiles and hepatic enzyme activities in rats. *British Journal of Nutrition, 94,* 526-532.

Kanner, J. (1994). Oxidative processes in meat and meat products: quality implications. *Meat Science, 36,* 169-189.

Kaur, G., Begg, D. P., Barr, D., Garg, M., Cameron-Smith, D., & Sinclair, A. J. (2010). Short-term docosapentaenoic acid (22: 5n-3) supplementation increases tissue docosapentaenoic acid, DHA and EPA concentrations in rats. *British Journal of Nutrition, 103,* 32-37.

Kearney, J. M. & McElhone, S. (1999). Perceived barriers in trying to eat healthier: results of a pan-EU consumer attitudinal survey. *British Journal of Nutrition, 81,* S133-S137.

Kilic, B., Cassens, R. G., & Borchert, L. L. (2002). Effect of turkey meat, phosphate, sodium lactate, carrageenan, and konjac on residual nitrite in cured meats. *Journal of Food Science, 67,* 29-31.

Kitessa, S. M., Young, P., Nattrass, G., Gardner, G., Pearce, K., & Pethick, D. W. (2012). When balanced for precursor fatty acid supply echium oil is not superior to linseed oil in enriching lamb tissues with long-chain n-3 PUFA. *British Journal of Nutrition, 108,* 71-79.

Kitts, D. D. (1997). An evaluation of the multiple effects of the antioxidant vitamins. *Trends in Food Science & Technology, 8,* 198-203.

Krause, B. L., Sebranek, J. G., Rust, R. E., & Mendonca, A. (2011). Incubation of curing brines for the production of ready-to-eat, uncured, no-nitrite-or-nitrate-added, ground, cooked and sliced ham. *Meat Science, 89,* 507-513.

Kumarasamy, Y., Cox, P. J., Jaspars, M., Rashid, M. A., & Sarker, S. D. (2003). Bioactive flavonoid glycosides from the seeds of *Rosa canina*. *Pharmaceutical Biology, 41,* 237-242.

Kuo, W. N., Kreahling, J. M., Shanbhag, V. P., Shanbhag, P. P., & Mewar, M. (2000). Protein nitration. *Molecular and Cellular Biochemistry*, *214,* 121-129.

Lauridsen, C., Buckley, D. J., & Morrissey, P. A. (1997). Influence of dietary fat and vitamin E supplementation on α–tocopherol levels and fatty acid profiles in chicken muscle membranal fractions and on susceptibility to lipid peroxidation. *Meat Science, 46,* 9-22.

Lecumberri, E., Goya, L., Mateos, R., Alía, M., Ramos, S., Izquierdo-Pulido, M. et al. (2007). A diet rich in dietary fiber from cocoa improves lipid profile and reduces malondialdehyde in hypercholesterolemic rats. *Nutrition, 23,* 332-341.

Lee, H., Kizito, S. A., Weese, S. J., CraigΓÇÉSchmidt, M. C., Lee, Y., Wei, C. et al. (2003). Analysis of headspace volatile and oxidized volatile compounds in dha-enriched fish oil on accelerated oxidative storage. *Journal of Food Science, 68,* 2169-2177.

Lenihan-Geels, G., Bishop, K. S., & Ferguson, L. R. (2013). Alternative sources of omega-3 fats: can we find a sustainable substitute for fish? *Nutrients, 5,* 1301-1315.

Levine, R. L., Williams, J. A., Stadtman, E. P., & Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. In P. Lester (Ed.), *Methods in Enzymology Oxygen Radicals in Biological Systems Part C*, *233*, 346-357. Academic Press.

Liu, G. & Xiong, Y. L. (2000). Oxidatively induced chemical changes and interactions of mixed myosin, β-lactoglobulin and soy 7S globulin. *Journal of the Science of Food and Agriculture, 80,* 1601-1607.

Liu, Q., Lanari, M. C., & Schaefer, D. M. (1995). A review of dietary vitamin E supplementation for improvement of beef quality. *Journal of Animal Science, 73,* 3131-3140.

Lovegrove, J. A., Brooks, C. N., Murphy, M. C., Gould, B. J., & Williams, C. M. (1997). Use of manufactured foods enriched with fish oils as a means of increasing long-chain n-3 polyunsaturated fatty acid intake. *British Journal of Nutrition, 78,* 223-236.

Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods: a review. *Molecular Nutrition & Food Research, 55,* 83-95.

Lund, M. N., Hviid, M. S., & Skibsted, L. H. (2007). The combined effect of antioxidants and modified atmosphere packaging on protein and lipid oxidation in beef patties during chill storage. *Meat Science, 76,* 226-233.

Lundberg, J. O., Weitzberg, E., & Gladwin, M. T. (2008). The nitrate–nitrite-nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery, 7,* 156-167.

Madsen, L. H. & Bertelsen, G. (1995). Spices as antioxidants. *Trends in Food Science & Technology, 6,* 271-277.

Maertens, L., Perez, J., Villamide, M., Cervera, C., Gidenne, T., & Xiccato, G. (2002). Nutritive value of raw materials for rabbits Egran Tables 2002. *World Rabbit Science, 10,* 157-166.

Magrinya, N., Bou, R., Rius, N., Codony, R., & Guardiola, F. (2012). Effect of fermentation time and vegetable concentrate addition on quality parameters of organic botifarra catalana, a cured-cooked sausage. *Journal of Agricultural and Food Chemistry, 60,* 6882-6890.

Mahfouz, M. M. & Kummerow, F. A. (2000). Cholesterol-rich diets have different effects on lipid peroxidation, cholesterol oxides, and antioxidant enzymes in rats and rabbits. *The Journal of Nutritional Biochemistry, 11,* 293-302.

Mancini, R. A. & Hunt, M. C. (2005). Current research in meat color. *Meat Science, 71,* 100-121.

Mantzioris, E., Cleland, L. G., Gibson, R. A., Neumann, M. A., Demasi, M., & James, M. J. (2000). Biochemical effects of a diet containing foods enriched with n-3 fatty acids. *The American Journal of Clinical Nutrition, 72,* 42-48.

Marriott, N. G., Garrett, J. E., Sims, M. D., & Abril, J. R. (2002). Performance characteristics and fatty acid composition of pigs fed a diet with docosahexaenoic acid. *Journal of Muscle Foods, 13,* 265-277.

Marriott, N. G., Garrett, J. E., Sims, M. D., Wang, H., & Abril, J. R. (2002). Characteristics of pork with docosahexaenoic acid supplemented in the diet. *Journal of Muscle Foods, 13,* 253-263.

Martinaud, A., Mercier, Y., Marinova, P., Tassy, C., Gatellier, P., & Renerre, M. (1997). Comparison of oxidative processes on myofibrillar proteins from beef during maturation and by different model oxidation systems. *Journal of Agricultural and Food Chemistry, 45,* 2481-2487.

Massey, L. K. (2002). Chapter 34 - Polyethylene Overview. In *Permeability Properties of Plastics and Elastomers (Second Edition)*, 209-215. Norwich, NY: William Andrew Publishing.

McAnulty, S. R., Nieman, D. C., Fox-Rabinovich, M., Duran, V., Mcanulty, L. S., Henson, D. A. et al. (2010). Effect of n-3 fatty acids and antioxidants on oxidative stress after exercise. *Medicine and Science in Sports and Exercise, 42,* 1704-1711.

Meadus, W. J., Duff, P., Uttaro, B., Aalhus, J. L., Rolland, D. C., Gibson, L. L. et al. (2009). Production of docosahexaenoic acid (DHA) enriched bacon. *Journal of Agricultural and Food Chemistry, 58,* 465-472.

Means, G. E. & Feeney, R. E. (1998). Chemical modifications of proteins: a review. *Journal of Food Biochemistry, 22,* 399-426.

Mercier, Y., Gatellier, P., & 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.

Meynier, A., Genot, C., & Gandemer, G. (1998). Volatile compounds of oxidized pork phospholipids. *Journal of the American Oil Chemists' Society, 75,* 1-7.

Mo, H., Zhu, Y., & Chen, Z. (2008). Microbial fermented tea - a potential source of natural food preservatives. *Trends in Food Science & Technology*, 19, 124-130.

Moghadasian, M. H. (2002). Experimental atherosclerosis: a historical overview. *Life Sciences, 70,* 855-865.

Morrissey, P. A., Buckley, D. J., Sisk, H., Lynch, P. B., & Sheehy, P. J. A. (1996). Uptake of α–tocopherol in porcine plasma and tissues. *Meat Science, 44,* 275-283.

Morrissey, P. A., Sheehy, P. J. A., Galvin, K., Kerry, J. P., & Buckley, D. J. (1998). Lipid stability in meat and meat products. *Meat Science, 49,* S73-S86.

Morrissey, P. A. & Tichivangana, J. Z. (1985). The antioxidant activities of nitrite and nitrosylmyoglobin in cooked meats. *Meat Science, 14,* 175-190.

Musella, M., Cannata, S., Rossi, R., Mourot, J., Baldini, P., & Corino, C. (2009). Omega-3 polyunsaturated fatty acid from extruded linseed influences the fatty acid composition and sensory characteristics of dry-cured ham from heavy pigs. *Journal of Animal Science, 87,* 3578-3588.

Narayan, B., Miyashita, K., & Hosakawa, M. (2006). Physiological effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-A review. *Food Reviews International, 22,* 291-307.

Nicolescu, A. C., Reynolds, J. N., Barclay, L. R., & Thatcher, G. R. (2004). Organic nitrites and NO: inhibition of lipid peroxidation and radical reactions. *Chemical Research in Toxicology, 17,* 185-196.

Nieto, G. & Ros, G. (2012). Modification of fatty acid composition in meat through diet: effect on lipid peroxidation and relationship to nutritional quality: a review. In: *Biochemistry, Genetics and Molecular Biology*, 953-978. ed. Catala.

Niki, E., Noguchi, N., Tsuchihashi, H., & Gotoh, N. (1995). Interaction among vitamin C, vitamin E, and beta-carotene. *The American Journal of Clinical Nutrition, 62,* 1322S-1326S.

Nissen, L. R., Byrne, D. V., Bertelsen, G., & Skibsted, L. H. (2004). The antioxidative activity of plant extracts in cooked pork patties as evaluated by descriptive sensory profiling and chemical analysis. *Meat Science, 68,* 485-495.

Okayama, T., Fujii, M., & Yamanoue, M. (1991). Effect of cooking temperature on the percentage colour formation, nitrite decomposition and sarcoplasmic protein denaturation in processed meat products. *Meat Science, 30,* 49-57.

Onibi, G. E., Scaife, J. R., Murray, I., & Fowler, V. R. (2000). Supplementary α–tocopherol acetate in full–fat rapeseed–based diets for pigs: influence on tissue α–tocopherol content, fatty acid profiles and lipid oxidation. *Journal of the Science of Food and Agriculture, 80,* 1625-1632.

Ordóñez, J. A., Hierro, E. M., Bruna, J. M., & Hoz, L. d. l. (1999). Changes in the components of dry-fermented sausages during ripening. *Critical Reviews in Food Science and Nutrition, 39,* 329-367.

Park, D., Xiong, Y. L., & Alderton, A. L. (2006). Concentration effects of hydroxyl radical oxidizing systems on biochemical properties of porcine muscle myofibrillar protein. *Food Chemistry, 101,* 1239-1246.

Parthasarathy, D. K. & Bryan, N. S. (2012). Sodium nitrite: The "cure" for nitric oxide insufficiency. *Meat Science, 92,* 274-279.

Pastorelli, G., Magni, S., Rossi, R., Pagliarini, E., Baldini, P., Dirinck, P. et al. (2003). Influence of dietary fat, on fatty acid composition and sensory properties of dry-cured Parma ham. *Meat Science, 65,* 571-580.

Pateiro, M., Lorenzo, J. M., Amado, I. R., & Franco, D. (2014). Effect of addition of green tea, chestnut and grape extract on the shelf-life of pig liver pâté. *Food Chemistry, 147,* 386-394.

Pegg, R. B. & Shahidi, F. (1997). Unraveling the chemical identity of meat pigments. *Critical Reviews in Food Science and Nutrition, 37,* 561-589.

Perlo, F., Gago-Gago, A., Rosmini, M., Cervera-Perez, R., Perez-Alvarez, J., Pagan-Moreno, M. et al. (1995). Modification of physico-chemical and colour parameters during the marketing of paté. *Meat Science, 41,* 325-333.

Pokorny, J., Yanishlieva, N., & Gordon, M. (2001). *Antioxidants in Food: Practical Applications*, 1-380. Woodhead Publishing Limited, Abington Hall, Abington.

Raes, K., De Smet, S., & Demeyer, D. (2001). Effect of double-muscling in Belgian Blue young bulls on the intramuscular fatty acid composition with emphasis on conjugated linoleic acid and polyunsaturated fatty acids. *Animal Science, 73,* 253-260.

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.

Ranken, M. D. (1981). The use of ascorbic acid in meat processing. In J. N. Counsell & Hornig D.H. (Eds.), *Vitamin C (Ascorbic Acid)*, 105-122. London: Aplied Science Publishers Ltd.

Requena, J. R., Chao, C. C., Levine, R. L., & Stadtman, E. R. (2001). Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proceedings of the National Academy of Sciences, 98,* 69-74.

Rey, A. I., Kerry, J. P., Lynch, P. B., Lopez-Bote, C. J., Buckley, D. J., & Morrissey, P. A. (2001). Effect of dietary oils and alpha-tocopheryl acetate supplementation on lipid (TBARS) and cholesterol oxidation in cooked pork. *Journal of Animal Science, 79,* 1201-1208.

Rice-evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M., & Pridham, J. B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research, 22,* 375-383.

Risé, P., Camera, M., Caruso, D., Ghezzi, S., Visioli, F., & Galli, C. (2004). Synthesis of long-chain polyunsaturated fatty acids is inhibited in vivo in hypercholesterolemic rabbits and in vitro by oxysterols. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 71,* 79-86.

Rodriguez-Carpena, J. G., Morcuende, D., & Estévez, M. (2011). Avocado by-products as inhibitors of color deterioration and lipid and protein oxidation in raw porcine patties subjected to chilled storage. *Meat Science 89,* 166-173.

Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S. et al. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *Journal of Biological Chemistry, 269,* 26066-26075.

Ruiz, J., Ventanas, J., Cava, R., Andrés, A., & García, C. (1999). Volatile compounds of dry-cured Iberian ham as affected by the length of the curing process. *Meat Science, 52,* 19-27.

Ruxton, C. H. S., Reed, S. C., Simpson, M. J. A., & Millington, K. J. (2004). The health benefits of omega–3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition and Dietetics, 17,* 449-459.

Rymer, C., Gibbs, R. A., & Givens, D. I. (2010). Comparison of algal and fish sources on the oxidative stability of poultry meat and its enrichment with omega-3 polyunsaturated fatty acids. *Poultry Science, 89,* 150-159.

Sahoo, J. & Anjaneyulu, A. S. R. (1997). Quality improvement of ground buffalo meat by preblending with sodium ascorbate. *Meat Science, 46,* 237-247.

Sales, J. & Koukolova, V. (2011). Dietary vitamin E and lipid and color stability of beef and pork: Modeling of relationships. *Journal of Animal Science, 89,* 2836-2848.

Sammet, K., Duehlmeier, R., Sallmann, H. P., Von Canstein, C., Von Mueffling, T., & Nowak, B. (2006). Assessment of the antioxidative potential of dietary supplementation with α-tocopherol in low-nitrite salami-type sausages. *Meat Science, 72,* 270-279.

Santos, C., Hoz, L., Cambero, M. I., Cabeza, M. C., & Ordóñez, J. A. (2008). Enrichment of dry-cured ham with α–linolenic acid and α–tocopherol by the use of linseed oil and α–tocopheryl acetate in pig diets. *Meat Science, 80,* 668-674.

Sardi, L., Martelli, G., Lambertini, L., Parisini, P., & Mordenti, A. (2006). Effects of a dietary supplement of DHA-rich marine algae on Italian heavy pig production parameters. *Livestock Science, 103,* 95-103.

Sárraga, C., Guárdia, M. D., Díaz, I., Guerrero, L., Garcia Regueiro, J. A., & Arnau, J. (2007). Nutritional and sensory quality of porcine raw meat, cooked ham and dry-cured shoulder as affected by dietary enrichment with docosahexaenoic acid (DHA) and α–tocopheryl acetate. *Meat Science, 76,* 377-384.

Sebranek, J. G., Cassens, R. G., Hoekstra, W. G., Winder, W. C., Podebradsky, E. V., & Kielsmeier, E. W. (1973). 15N tracer studies of nitrite added to a comminuted meat product. *Journal of Food Science, 38,* 1220-1223.

Sebranek, J. G. & Bacus, J. N. (2007). Cured meat products without direct addition of nitrate or nitrite: what are the issues? *Meat Science, 77,* 136-147.

Shacter, E. (2000). Quantification and significance of protein oxidation in biological samples. *Drug metabolism reviews, 32,* 307-326.

Shull, S., Heintz, N. H., Periasamy, M., Manohar, M., Janssen, Y. M., Marsh, J. P. et al. (1991). Differential regulation of antioxidant enzymes in response to oxidants. *Journal of Biological Chemistry, 266,* 24398-24403.

Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. *The American Journal of Clinical Nutrition, 70,* 560S-569S.

Simpson, C. A. & Sofos, J. N. (2009). Antimicrobial ingredients. In *Ingredients in Meat Products*, 301-377. New York: Springer Science+Business Media LLC.

Sindelar, J. J., Cordray, J. C., Sebranek, J. G., Love, J. A., & Ahn, D. U. (2007a). Effects of varying levels of vegetable juice powder and incubation time on color, residual nitrate and nitrite, pigment, ph, and trained sensory attributes of ready-to-eat uncured ham. *Journal of Food Science, 72,* S388-S395.

Sindelar, J. J., Cordray, J. C., Sebranek, J. G., Love, J. A., & Ahn, D. U. (2007b). Effects of vegetable juice powder concentration and storage time on some chemical and sensory quality attributes of uncured, emulsified cooked sausages. *Journal of Food Science, 72,* S324-S332.

Sindelar, J. J. & Milkowski, A. L. (2011). Sodium nitrite in processed meat and poultry meats: A review of curing and examining the risk/benefit of its use. *American Meat Science Association White Paper Series*, *3*, 1-16.

Sioen, I., Matthys, C., De Backer, G., Van Camp, J., & Henauw, S. D. (2007). Importance of seafood as nutrient source in the diet of Belgian adolescents. *Journal of Human Nutrition and Dietetics, 20,* 580-589.

Sioen, I. A., Pynaert, H., De Backer, G., Van Camp, J., & De Henauw, S. (2006). Dietary intakes and food sources of fatty acids for Belgian women, focused on n-6 and n-3 polyunsaturated fatty acids. *Lipids, 41,* 415-422.

Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide, 24,* 176-183.

Sobotka, W., Flis, M., Antoszkiewicz, Z., Lipínski, K., & Zdunczyk, Z. (2012). Effect of oat by-product antioxidants and vitamin E on the oxidative stability of pork from pigs fed diets supplemented with linseed oil. *Archives of Animal Nutrition, 66,* 27-38.

Soto, E., Hoz, L., Ordóñez, J. A., Hierro, E., Herranz, B., López-Bote, C. et al. (2008). Impact of feeding and rearing systems of Iberian pigs on volatile profile and sensory characteristics of dry-cured loin. *Meat Science, 79,* 666-676.

Souza, J. M., Peluffo, G., & Radi, R. (2008). Protein tyrosine nitration-functional alteration or just a biomarker? *Free Radical Biology and Medicine, 45,* 357-366.

Specht, K. & Baltes, W. (1994). Identification of volatile flavor compounds with high aroma values from shallow-fried beef. *Journal of Agricultural and Food Chemistry, 42,* 2246-2253.

Sprecher, H., Luthria, D. L., Mohammed, B. S., & Baykousheva, S. P. (1995). Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *Journal of Lipid Research, 36,* 2471-2477.

Stadtman, E. R. & Levine, R. L. (2000). Protein oxidation. *Annals of the New York Academy of Sciences, 899,* 191-208.

Stadtman, E. R. & Levine, R. L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids, 25,* 207-218.

Stagsted, J., Bendixen, E. k., & Andersen, H. J. (2004). Identification of specific oxidatively modified proteins in chicken muscles using a combined immunologic and proteomic approach. *Journal of Agricultural and Food Chemistry, 52,* 3967-3974.

Stocker, R. & Keaney, J. F. (2005). New insights on oxidative stress in the artery wall. *Journal of Thrombosis and Haemostasis, 3,* 1825-1834.

Sullivan, G. A. & Sebranek, J. G. (2012). Nitrosylation of myoglobin and nitrosation of cysteine by nitrite in a model system simulating meat curing. *Journal of Agricultural and Food Chemistry, 60,* 1748-1754.

Superior Health Council of Belgium (2006). Voedingsaanbevelingen voor België, Brussel, Belgium, nr. 7145-2. *Hoge Gezondheidsraad.*

Tang, S. Z., Kerry, J. P., Sheehan, D., Buckley, D. J., & Morrissey, P. A. (2000). Dietary tea catechins and iron-induced lipid oxidation in chicken meat, liver and heart. *Meat Science, 56,* 285-290.

Tanghe, S., Millet, S., & De Smet, S. (2013). Echium oil and linseed oil as alternatives for fish oil in the maternal diet: Blood fatty acid profiles and oxidative status of sows and piglets. *Journal of Animal Science, 91,* 3253-3264.

Tarladgis, B. G., Watts, B. M., Younathan, M. T., & Dugan Jr, L. (1960). A distillation method for the quantitative determination of malonaldehyde in rancid foods. *Journal of the American Oil Chemists Society, 37,* 44-48.

Terns, M. J., Milkowski, A. L., Rankin, S. A., & Sindelar, J. J. (2011). Determining the impact of varying levels of cherry powder and starter culture on quality and sensory attributes of indirectly cured, emulsified cooked sausages. *Meat Science, 88,* 311-318.

Tiago, T., Aureliano, M., & Gutiérrez-Merino, C. (2008). Effects of reactive oxygen and nitrogen species on actomyosin and their implications for muscle contractility. In C. Gutiérrez-Merino & C. Leeuwenburgh (Eds.), *Free Radicals in Biology and Medicine*, 1-19.

Trefan, L., Bünger, L., Bloom-Hansen, J., Rooke, J. A., Salmi, B., Larzul, C. et al. (2011). Meta-analysis of the effects of dietary vitamin E supplementation on α-tocopherol concentration and lipid oxidation in pork. *Meat Science, 87,* 305-314.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chemistry, 99,* 835-841.

USDA (1995). Processing inspector's calculations handbook (FSIS Directive 7620.3). http://www.fsis.usda.gov/OPPDE/rdad/FSISDirectives/7620-3.pdf [Electronic version].

Utrera, M., Morcuende, D., Rodriguez-Carpena, J. G., & Estévez, M. (2011). Fluorescent HPLC for the detection of specific protein oxidation carbonyls - a-aminoadipic and g-glutamic semialdehydes - in meat systems. *Meat Science 89,* 500-506.

Valencia, I., Ansorena, D., & Astiasarán, I. (2006). Nutritional and sensory properties of dry fermented sausages enriched with n-3 PUFAs. *Meat Science, 72,* 727-733.

Valencia, I., Ansorena, D., & Astiasarán, I. (2007). Development of dry fermented sausages rich in docosahexaenoic acid with oil from the microalgae *Schizochytrium* sp.: Influence on nutritional properties, sensorial quality and oxidation stability. *Food Chemistry, 104,* 1087-1096.

Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J., & De Smet, S. (2014). Nitrite-curing of chicken, pork and beef inhibits oxidation but does not affect NOC-specific DNA adduct formation during in vitro digestion. *Journal of Agricultural and Food Chemistry, 62*, 1980-1988.

Venegas-Caleón, M., Sayanova, O., & Napier, J. A. (2010). An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. *Progress in lipid research, 49,* 108-119.

Ventanas, S., Estévez, M., Tejeda, J. F., & Ruiz, J. (2006). Protein and lipid oxidation in *Longissimus dorsi* and dry cured loin from Iberian pigs as affected by crossbreeding and diet. *Meat Science, 72,* 647-655.

Villaverde, A., Parra, V., & Estévez, M. (2014). Oxidative and nitrosative stress induced in myofibrillar proteins by a hydroxyl-radical generating system: impact of nitrite and ascorbate. *Journal of Agricultural and Food Chemistry*, *62*, 2158-2164.

von Schacky, C. & Weber, P. C. (1985). Metabolism and effects on platelet function of the purified eicosapentaenoic and docosahexaenoic acids in humans. *Journal of Clinical Investigation, 76,* 2446-2450.

Vossen, E., Doolaege, E. H., Moges, H. D., De Meulenaer, B., Szczepaniak, S., Raes, K., & De Smet, S. (2012a). Effect of sodium ascorbate dose on the shelf life stability of reduced nitrite liver pâtés. *Meat Science, 91,* 29-35.

Vossen, E., Utrera, M., De Smet, S., Morcuende, D., & Estévez, M. (2012b). Dog rose (*Rosa canina* L.) as a functional ingredient in porcine frankfurters without added sodium ascorbate and sodium nitrite. *Meat Science, 92,* 451-457.

Wakamatsu, J., Nishimura, T., & Hattori, A. (2004). A Zn-porphyrin complex contributes to bright red color in Parma ham. *Meat Science, 67,* 95-100.

Walczewska, A., Dziedzic, B., Stepien, T., Swiatek, E., & Nowak, D. (2010). Effect of dietary fats on oxidative-antioxidative status of blood in rats. *Journal of Clinincal Biochemistry and Nutrition, 47,* 18-26.

Warnants, N., Van Oeckel, M. J., & Boucqué, C. (1998). Effect of incorporation of dietary polyunsaturated fatty acids in pork backfat on the quality of salami. *Meat Science, 49,* 435-445.

Williams, C. M. (2000). Dietary fatty acids and human health. *Annales de Zootechnie, 3,* 165-180.

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

Woolford, G., Cassens, R. G., Greaser, M. L., & Sebranek, J. G. (1976). The fate of nitrite: reaction with protein. *Journal of Food Science, 41,* 585-588.

World Cancer Research Fund (2011). Second expert report 2011 - Food, nutrition, physical activity and the prevention of cancer: a global perspective. *http://www.dietandcancerreport.org/expert_report/report_contents/index.php,* Accessed 26-03/2014.

World Health Organization (2003). *Diet, nutrition and the prevention of chronic diseases: report of a joint WHO/FAO expert consultation*. (916 ed.) World Health Organization.

Yang, H., Zhang, Y., & Pöschl, U. (2010). Quantification of nitrotyrosine in nitrated proteins. *Analytical and Bioanalytical Chemistry, 397,* 879-886.

Yanishlieva, N. V., Marinova, E., & Pokorny, J. (2006). Natural antioxidants from herbs and spices. *European Journal of Lipid Science and Technology, 108,* 776-793.

Yen, G. C., Duh, P. D., & Tsai, H. L. (2002). Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chemistry, 79,* 307-313.

Zapata, S. & Dufour, J. P. (1992). Ascorbic, dehydroascorbic and isoascorbic acid simultaneous determinations by reverse phase ion interaction HPLC. *Journal of Food Science, 57,* 506-511.

Zhang, W., Xiao, S., & Ahn, D. U. (2012). Protein oxidation: basic principles and implications for meat quality. *Critical Reviews in Food Science and Nutrition, 53,* 1191-1201.

Zheng, S., Qian, Z., Sheng, L., & Wen, N. (2006). Crocetin attenuates atherosclerosis in hyperlipidemic rabbits through inhibition of LDL oxidation. *Journal of cardiovascular pharmacology, 47,* 70-76.

Zuo, Y., Wang, C., & Van, T. (2006). Simultaneous determination of nitrite and nitrate in dew, rain, snow and lake water samples by ion-pair high-performance liquid chromatography. *Talanta, 70,* 281-285.

BEKNOPTE BIOGRAFIE

Els Vossen (°Gent, 7 Maart 1983) behaalde in 2001 het diploma hoger secundair onderwijs (Moderne Talen - Wiskunde 8u) aan het Sint-Gertrudiscollege te Wetteren. In 2006 studeerde zij met onderscheiding af als Bio-Ingenieur in de Landbouwkunde aan de Faculteit Bio-Ingenieurswetenschappen van de Universiteit Gent. Sinds Augustus 2006 is ze werkzaam als assistent aan de Vakgroep Dierlijke Productie. Als onderzoeksactiviteiten werkte ze mee aan verschillende lopende projecten op de vakgroep, m.b.t. het optimaliseren van de oxidatieve stabiliteit en nutritionele kwaliteit van vlees en vleesproducten in relatie tot recente voedingsaanbevelingen en tendensen in de vleesindustrie. Daarnaast stond ze in voor de begeleiding van studenten bij het uitvoeren van hun bachelorproef of masterscriptie en was ze verantwoordelijk voor diverse practica en excursies. Zij is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en nam deel aan nationale en internationale congressen waar ze diverse voordrachten en postervoorstellingen verzorgde. In 2007 behaalde ze het diploma van Geaggregeerde voor het Secundair Onderwijs (AILO opleiding) en in 2014 slaagde ze voor de doctoraatsopleiding van de Doctoral School of (Bioscience) Engineering.

EDUCATION

1994-1997	<u>Scuola Europea</u>, Varese, Italy (ASO Dutch Department)
1997-2001	<u>Sint-Gertrudiscollege</u>, Wetteren, Belgium
	(ASO: Modern languages-Mathematics (8h)
2001-2006	<u>Bioscience Engineering, Agricultural Sciences</u>, Faculty of Bioscience Engineering, Ghent University (Belgium). Graduated with distinction.
	<u>Master thesis:</u> *Effect of n-3 fatty acids enriched meat on oxidative stress and atherosclerosis, using a rabbit as animal model* (score 15/20) Laboratory of animal nutrition and animal product quality; Department of Animal Production, Faculty of Bioscience Engineering, Ghent University. Promoter: Prof. dr. ir. K. Raes
2006	<u>Application to Doctoral fellowship</u> granted by IWT (score 15/20)
2006-2007	<u>Teacher Training Programme (AILO)</u>, Faculty of Bioscience Engineering, Ghent University. Graduated with distinction.
2007-2014	<u>Doctoral School</u> of (Bioscience) Engineering
2009	<u>Advanced Course in Meat Science</u>, PhD course International Congress of Meat Science and Technology, 12-16/08/2009, University of Copenhagen, Copenhagen, Denmark.
2011	<u>Advanced Course in Meat Science</u>, PhD course International Congress of Meat Science and Technology, 3-6/08/2011, Ghent University, Ghent,Belgium.

PROFESSIONAL ACTIVITIES

2007-present: <u>Assistant</u> at laboratory of animal nutrition and animal product quality, Department of Animal Production, Faculty of Bioscience Engineering, Ghent University. Promoter: Prof. dr. ir. Stefaan De Smet

<u>Main tasks:</u>
- Performing research on the topic of '*Oxidative stability and nutritional quality of enriched meat'*
- Close involvement in different research topics in the laboratory (lowering nitrite content in meat products, protein oxidation in dry fermented sausages, enrichment of meat products with n-3 fatty acids, oxidation processes during *in vitro* digestion of meat)
- Supervision of master thesis students
- Assistant teaching (practical exercises and excursions) of several courses (meat science, animal production systems)

2010: Research activities (6 weeks) at the Animal production and Food Science, Food Technology Department, Faculty of Veterinary Science, University of Extremadura, Cáceres, Spain

PUBLICATIONS

Peer reviewed as first or co-author

Raes, K., Doolaege, E.H.A., Deman, S., Vossen, E., De Smet, S. Effect of carnosic acid, quercetin and α-tocopherol on lipid and protein oxidation in an *in vitro* simulated gastric digestion model. International Journal of Food Sciences and Nutrition, accepted (20/08/2014).

Callewaert, C., Buysschaert, B., Vossen, E., Fievez, V., Van de Wiele, T., Boon, N. (2014). Development of a novel artificial sweat composition. Journal of Microbial Methods, 103: 6-8.

Van Hecke, T., Vossen, E., Vanden Bussche, J., Raes, K, Vanhaecke, L., De Smet, S. (2014). Fat content and nitrite-curing influence the formation of oxidation products and NOC-specific DNA adducts during *in vitro* digestion of meat. Plos One, 9: doi:10.1371/journal.pone.0101122

Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J., De Smet, S. (2013). Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during *in vitro* digestion. Journal of Agricultural and Food Chemistry, 62: 1980-1988.

Vossen, E., Utrera, M., De Smet, S., Morcuende, D., Estévez, M. (2012). Dog rose (Rosa canina L.) as a functional ingredient in porcine frankfurters without added sodium ascorbate and sodium nitrite. Meat Science, 92: 451–457.

Vossen, E., Doolaege, E.H.A., Moges, H.D., De Meulenaer, B., Szczepaniak, S., Raes, K. De Smet, S. (2012). Effect of sodium ascorbate dose on the shelf life stability of reduced nitrite liver pâté. Meat Science, 91: 29-35.

Doolaege, E.H.A., Vossen, E., Raes, K., De Meulenaer, B., Verhe, R., De Smet, S. (2012) Effect of rosemary extract dose on lipid oxidation, colour stability and antioxidant concentrations, in reduced nitrite liver pâtés. Meat Science, 90: 925–931 (equal contribution).

Vossen, E., Raes, K., Maertens, L., Vandenberge, V., Haak, L., Chiers, K., Ducatelle, R., De Smet S. (2012). Diets containing n-3 fatty acids enriched pork: effect on blood lipids, oxidative status and atherosclerosis in rabbits. Journal of Food Biochemistry, 36: 359-268.

Vossen, E., Ntawubizi, M., Raes, K., Smet, K., Huyghebaert, G., Arnouts, S., De Smet, S. (2010). Effect of dietary antioxidant supplementation on the oxidative status of plasma in broilers. Journal of Animal Physiology and Animal Nutrition, 95: 198-205.

Under review
Claeys, E., Vossen, E., De Smet, S. Determination of alpha-tocopherol by reversed phase HPLC in animal-derived foods and in feed without saponification. Under review (submitted 20/08/2014 to Journal of the Science of Food and Agriculture).

Van Hecke, T., Vossen, E., Hemeryck, L., Vanden Bussche, J., Vanhaecke, L., De Smet, S. Increased Oxidative and Nitrosative Reactions During Digestion Could Contribute to the Association between Well-done Red Meat Consumption and Colorectal Cancer. Under review (submitted 03/07/2014 to Food Chemistry).

Gadeyne, F., Van Ranst, G., Vlaeminck, B., Vossen, E., Van der Meeren, P., Fievez, V. Protection of poly-unsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover. Under review (submitted 18/02/2014 to Food Chemistry).

Conference proceedings

Vossen, E., Gevaert, K., De Smet, S. (2013). Effect of sodium nitrite on protein oxidation in pork myofibrillar proteins. Proceedings 59th International Congress of Meat Science and Technology, Izmir, Turkey, 18-23/08/2013 (poster presentation).

Van Hecke, T., Vossen, E., Vanden Bussche, J., Vanhaecke, I., De Smet, S. (2013). Fat, cooking method and nitrite-curing of meat influences formation of malondialdehyde and NOC-specific DNA adducts during *in vitro* digestion. Proceedings 59th International Congress of Meat Science and Technology, Izmir, Turkey, 18-23/08/2013 (poster presentation).

Van Hecke, T., Vanden Bussche, J., Vanhaecke, I., Vossen, E., De Smet, S. (2013). The effect of nitrite-curing of chicken, pork and beef on oxidation and NOC-specific DNA adduct formation during *in vitro* digestion. Proceedings 59th Icomst, Izmir, Turkey, 18-23/08/2013 (poster presentation).

Vossen, E., Utrera, M., De Smet, S., Morcuende, D., Estévez, M. (2012). Dog rose (Rosa canina L.) as a functional ingredient in porcine frankfurters without added sodium ascorbate and sodium nitrite. Proceedings 57th International Congress of Meat Science and Technology, Ghent, Belgium, 7-12/08/2011. Short paper 019 (oral presentation).

Callewaert, C., Buysschaert, B., Vossen, E., Van Gele, M., Van de Wiele, T., Boon, N. (2013) The SCIN simulation: a novel *in vitro* technique to study microbial colonization in the axillary region. Applied Biological Sciences, 18th National symposium, Abstracts.

Vossen, E., Van Mullem, D., Raes, K., De Smet, S. (2010). Fatty acid composition and sensory acceptability of dry cured ham influenced by linseed oil, fish oil or microalgae included in the pig feed. Proceedings 56th International Congress of Meat Science and Technology, Jeju, Korea, 15-20/08/2010. Short paper B017 (poster presentation).

Vossen, E., Van Mullem, D., Raes, K., De Smet, S. (2009). Fatty acid profile of Meat and Fermented Sausages influenced by including Linseed, Fish oil or Microalgae in Pig Feed.

Proceedings 55th International Congress of Meat Science and Technology, Copenhagen, Denmark, 16-21/08/2009. Short paper PE9.30 (poster presentation).

Vossen, E., Haak, L., Van Der Eecken, E., Maertens, L., Chiers, K., De Smet S., Raes, K. (2009). Effect of dietary incorporation of n-3 fatty acid enriched pork meat on the fatty acid profile, oxidative status, blood lipids and atherosclerosis in the rabbit as experimental model. Belgian Nutrition Society, Brussel, Belgium, 23/04/2009 (oral presentation).

Pappijn, M., Vossen, E., Missotten, J.A.M., Raes, K. & De Smet, S. (2008). The influence of sow dietary polyunsaturated fatty acid source on the oxidative status of piglets. 33rd NVO Meeting, 25 April 2008, The new Forum Building of Wageningen University, Wageningen, The Netherlands, p. 35-36.

Conferences, workshops, seminars

Vlav seminar: "Natuurlijke ingrediënten in de vleesindustrie" 20-04-2012, Sfinc, Eke-Nazareth, Belgium. Invited speaker.

Advanced Meat Science, PhD course International Congress of Meat Science and Technology, 3-6/08/2011, Ghent University, Ghent, Belgium.

BAMST studienamiddag, Vlees: duurzaam? 16/10/2012, Salons Roskam, Merelbeke, Melle, Belgium.

BAMST studienamiddag, World cancer research rapport: een uitdaging voor de vleesindustrie, 25/09/2010, ILVO, Melle, Belgium.

BAMST studienamiddag, Imago van vlees en vleesproducten, 08/10/2009, ILVO, Melle, Belgium.

Advanced Meat Science, PhD course International Congress of Meat Science and Technology, 12-16/08/2009, University of Copenhagen, Copenhagen, Denmark.

34th Animal Nutrition Researchers (ANR) Meeting. ILVO, 3/04/2009, Animal Sciences, Melle, Belgium.

3rd practical short course Speciality and Functional Oils Market Trends, Nutrition & Health, Utilization in Food Systems', 9-10/10/2008, Universiteit Gent, Gent, Belgium.

33rd NVO Meeting. The new Forum Building 25/04/2008, Wageningen University, Wageningen, The Netherlands.

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