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**The effects of dietary supplementation and  
product formulation on the oxidative stability of  
lipids and proteins in meat and meat products**

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## **1. Abstract**

Meat and processed meats are largely consumed in the western world, but lately there has been a great concern about their possible negative impacts on human health. Consequently, there is growing interest among food scientists to better understand the diverse chemical and microbial modifications that take place during processing and storage and that can compromise the nutritional characteristics and safety of such products. In the past decades, attention has been addressed towards the study of lipid oxidation and microbial deterioration in meat and meat products, whereas the occurrence and impact of protein oxidation has been disregarded. Since protein oxidation can take place in living tissues, scientists are currently interested in highlighting its influence on meat quality and human nutrition. Recent studies have contributed to solid scientific knowledge regarding basic protein oxidation mechanisms, and in several methodologies to assess such degradation process in food.

In accordance with this vision, the general objective of this PhD thesis was to study the effects of dietary supplementation, product formulation and storage conditions on the oxidative stability of lipids and proteins in meat and meat products, with the final aim to better understand the trends of these oxidative processes in such products in order to identify strategies to improve their overall quality.

For this purpose, various experiments were conducted. First, photooxidation of both lipid and protein fractions in raw meat stored under commercial retail conditions, was investigated. Beef and chicken meat slices were packed in vessels with transparent shrink film and exposed to white fluorescent light 4 °C for 0, 3 and 5 days (12-h light/12-h darkness cycle). Lipid oxidation was assessed by measuring peroxide value (PV) and thiobarbituric acid-reactive substances (TBARs), while the quantification of thiol and carbonyl groups were used to evaluate the extent of protein

oxidation. In general, both lipid and protein oxidation parameters significantly increased during storage at 4 °C under darkness conditions, with a greater impact when exposed to light. Beef meat exhibited much higher lipid and protein oxidation (about 12 and 2 times, respectively) than chicken breast. This study evidenced that storage time and exposure to light can greatly affect both lipid and protein oxidations in raw meats, especially beef meat, thus suitable strategies (i.e. film types and/or active packaging) should be found to improve their oxidative stability and reduce the impact of light exposure.

The second experiment focused on the effect of plant extracts mixture on the oxidative stability of chicken breast meat during chill and frozen storage. The experiment was set up according to a Randomized Complete Block Design, with 2 experimental groups and 12 replications per treatment. Male Ross 308 broilers were fed either a diet supplemented with or without plant extract mixture (0.35 g/kg feed) for 42 days. Freshly prepared samples were stored for short-term storage at 4 °C for 0 and 7 days, then packed in plastic bags under vacuum and subjected to long-term frozen storage at -18 °C for one year. No effect of the antioxidant plant extract mixture on lipid oxidation of fresh meat samples stored at 4 °C, was observed. After frozen storage at -18 °C for 1 year, samples obtained with dietary antioxidant supplementation had significantly ( $p < 0.05$ ) lower PV, TBARs and carbonyls contents and higher values of sulfhydryl level than the basal diet meat. These results suggest that dietary antioxidants can minimize the oxidative stability of both lipids and proteins in chicken breast meat during long-term frozen storage.

The third experiment aimed at evaluating the influence of partial or total replacement of pork back-fat (60% and 100%) by hempseed oil-in-water emulsion on the chemical composition, oxidative stability, and sensory analysis of reduced fat sausages enriched with n-3

polyunsaturated fatty acids (PUFA). The application of hempseed oil caused a significant reduction of saturated fatty acids, a significant increase in PUFA and a significant reduction in the n-6/n-3 ratio. The addition of hempseed oil to sausages caused a significant increase on PV, phytosterols and their oxidation products, but it did not significantly affect TBARs, thiol content, cholesterol and its oxidation products. Moreover, protein carbonyls significantly decreased with increasing amount of hempseed oil. Cooked sausages with 60% replacement showed good acceptability scores, similar to those of samples formulated with pork back fat (control). The usage of hempseed oil-in-water emulsion for partial replacement of pork back fat could be a good strategy to improve the nutritional properties of cooked sausages, without impairing their oxidative stability and sensory profile.

**Keywords:** lipid oxidation, protein oxidation, plants extracts, product formulation, plant oils

## **2. Introduction**

### **2.1. Meat production, consumption and health**

Meat and meat products constitute important sources of protein, fat, and several functional compounds, hence are considered a major part of the human diet with strong implications on health, economy, and culture worldwide. Global meat production and consumption have increased rapidly in recent decades, Pork is the most widely consumed meat in the world, followed by poultry, beef, and mutton. Poultry production is the fastest growing meat sector, increasing 4.7 percent in 2010 to 98 million tons (Dave and Ghaly, 2011). Worldwide, per capita meat consumption increased from 41.3 kg in 2009 to 41.9 kg in 2010 (Worldwatch Institute, 2011). People in the developing world eat 32 kg of meat a year on average, compared to 80 kg per person in the industrialized world (Worldwatch Institute, 2012).

Meat is a good source of dietary proteins in many countries and provides high biological value. Meat has an important place in a healthy diet, as it provides essential amino acids, fat, iron high bioavailability, vitamins (especially those of the B group), balanced proteins, and other essential minerals (such as zinc) (Givens et al., 2006, Franca, 2015). However, it may also represent some risks to human health. Depending on several factors, many reports warn against its metabolic deleterious effects specially linked to cholesterol and saturated fatty acids (SFA) levels. Low polyunsaturated fatty acids (PUFA) levels, or inappropriate SFA/PUFA or PUFA n-6/n-3, have been represented as an inconvenience in usual meat consumption. Thus, consumers often associate meat with a negative image as a high fat and cancer-promoting food (Pighin et al. 2016).

Actually, the effect of meat consumption is a controversial issue in human health and diet is supposed to be one of the most important contributing factors to cancer risk. For this reason,

the European Prospective Investigation into Cancer and Nutrition (EPIC), was devised, to investigate the relationship between diet, metabolic and genetic factors, and cancer (Bingham and Riboli, 2004). Recent studies have showed that there is a moderate positive association between processed meat consumption and mortality, in particular due to cardiovascular diseases, but also to cancer (Rohrmann et al., 2013). Furthermore, meta-analyses show that high consumers of cured meats and red meat have an increased risk of colorectal cancer (Aykan et al., 2015). Heme group catalyzes the formation of apparent total N-nitroso compounds (ATNC) and lipid peroxidation end products, which partially explains the promoting effect of red and processed meat on colorectal cancer (CRC). Several mechanisms may explain the effect of heme on CRC, and the 2 major hypotheses are: (i) catalyzes the endogenous formation of ATNC; and (ii) heme catalyzes the peroxidation of dietary fats. Calcium salts, chlorophyll, vitamin C, and several polyphenols may reduce these deleterious effects of heme (Bastide et al., 2011). ATNC collectively comprise nitrosyl iron and S-nitrosothiols, besides nitrosamines and nitrosamides (Bastide et al., 2011). In universal guidelines for healthy nutrition, the World Cancer Research Fund (WCRF) reported, at the end of 2007, “limit intake of red meat and avoid processed meat”, as a result of the convincing evidence for an association between processed meats intake and CRC development (Demeyer et al., 2008). Epidemiological and experimental evidence supports the hypothesis that heme iron present in meat promotes CRC. Evidences from human studies also suggest that increased dietary intake of heme iron is associated with increased incidences of CRC (Lee et al., 2004). These findings are in line with the recent results of Mosby et al. (2012), who indicate that heme group could increase lipid peroxidation, giving rise to mutagenic deoxyribonucleic acid (DNA) adducts. Therefore, the prospected correlation between high meat



intake and human health problems, such as obesity, cardio-vascular and cancer diseases, has led to a reduction in meat consumption (Schönfeldt, 2008).

Cardiovascular diseases (CVD) are one of the leading causes of death in men and women of all ethnic groups. Lower energy intake and differential consumption of multiple specific nutrients and foods are characteristic of individuals at low risk of CVD. Shay et al. (2012) reported that the identification of dietary habits associated with low risk of CVD is important for further development of public health efforts aimed at reduction/prevention of CVD. Micha et al. (2012) investigates the effects of processed (using sodium/other preservatives) and unprocessed (fresh/frozen) red meat consumption on coronary heart disease (CHD) and diabetes. The overall findings suggest that both types of meat consumption are not beneficial for cardiometabolic health, and that clinical and public health guidance should especially prioritize reducing processed meat consumption (Micha et al., 2012). Meat lipid fraction is quite controversial in this context. Consumption of saturated dietary fats and n-6 PUFA increases the CVD risk, whereas consumption of n-3 PUFA may reduce CVD incidence (Simopoulos, 1997). n-3 PUFA, in fact, have shown positive effects preventing and reducing the risks associated with CVD, rheumatoid arthritis, cancer, obesity and some other health problems (Simopoulos, 2004). Humans historically consumed a diet with a n-6:n-3 ratio of 1:1 to 4:1 (Eaton et al., 1996; Simopoulos, 2006). However, the typical ratio in developed countries is now greater than 10:1 (Azain, 2004). In contrast, a traditional Mediterranean diet yields a PUFA n-6/n-3 ratio of 2:1, which is much lower than for the aforementioned northern countries. Some authors however consider that assessing this ratio is irrelevant and that decreasing n-6 PUFA may be harmful (Szostak-Wegierek et al., 2013).

Regarding nutritional aspects and human health, poultry meat well fits the current consumer demand for a low-fat meat and a healthy fat source, as nutritional recommendations include the reduction of total fat, saturated fat and cholesterol consumption to prevent the incidence of most common chronic disorders (Barroeta et al., 2007). Therefore, there is a growing demand for food products with an appropriate content and profile of unsaturated FA, a trend that is currently influencing the production of poultry meat (Narciso-Gaytàn et al., 2010). Throughout the years, the poultry industry has changed and adapted to meet the consumer demands of meat products. In fact, the poultry industry is continuously focused on the development of food products with a modified FA profile, searching a larger n-3 FA content.

## **2.2 Oxidative quality and shelf-life of meats**

In nature, the progression of cells toward apoptosis and death is facilitated by oxidative processes that involve the formation of free radicals and their reactions within the tissue systems of animals and humans (Fiers et al., 1999). A free radical is a molecule that is able to exist in an independent state (that is, free) and carry unpaired electrons in the valence orbits (that is, radical). This condition is thermodynamically unfavorable and the molecules will attempt to reach a more stable state by reacting with another molecule, cleaving a hydrogen atom from a carbon–hydrogen bond, and donating or accepting electrons from neighboring compounds (Halliwell and Gutteridge 2007). This results in free radicals displaying a more behaviorally reactive chemical species than nonradical molecules. Mitochondria are regarded as the main source of free radical formation (Cadenas and Davis, 2000) in the tissue systems of living animals. The mitochondrial electron transport chain and its reactions generate superoxide anion radicals that can be subsequently converted to hydrogen peroxide. This can lead to the formation

of hydroxyl radicals that react with muscle and other tissue systems impacting the well-being or the performance of animals. Due to their highly reactive nature, free radicals exist in low concentrations ( $10^{-4}$  to  $10^{-9}$  M) and their effects are initiated locally where they are generated (Southorn and Powis, 1988). There are 2 possible outcomes for free radical reactions. The first is chemical modification of the surrounding compounds (such as oxidation of amino acids, lipids, or vitamins). This can promote loss of physiological function in living animals or initiation of undesirable changes in the muscle tissue system, leading to deterioration of quality attributes such as color stability, flavor, or nutritive characteristics after death. Second, the affected compound can become a radical, causing a series of electron exchanges among several molecules leading to DNA damage or oxidation of lipids and proteins.

### **2.2.1 Biological Effects of RS**

The damaging effects of oxidative processes are well recognized in the medical field and the food industry, for example, the involvement of lipid oxidation in the generation of reactive compounds, which causes damage to cellular materials. These effects have been implicated in a number of physiological disorders and diseases (Montine et al., 2004) and negative changes in meat quality (Kanner, 1994). Having noted these effects, it is important to understand that low levels of RS like  $H_2O_2$  can in some circumstances enable cells to survive otherwise lethal oxidative stress (Niki, 2012). Three significant changes take place due to increased oxidative changes; namely DNA damage or oxidation of lipids and proteins.

### **2.2.2 Chemistry behind lipid and protein oxidation**

Lipids are one of the most chemically unstable food components and will readily undergo oxidative reactions, driven by the degree of unsaturation of its fatty acids (FA) and the presence of catalysts and molecular oxygen (Min and Ahn, 2005). Lipid oxidation is defined as a free radical chain reaction and consists of three steps: initiation, propagation, and termination (Min and Ahn, 2005). In the initiation phase, a hydrogen atom is abstracted from a carbon neighbouring a double bond in an unsaturated FA (RH) to form a free radical lipid (alkyl radical, R•). The alkyl radical may react with molecular oxygen to generate diverse radical species, such as peroxy radicals (ROO•). These radicals may find stability during the subsequent propagation phase by abstracting a hydrogen atom from another susceptible molecule, including an adjacent RH creating a lipid hydroperoxide (ROOH) and a new R•. These propagation mechanisms may occur up to 100 times before two R• combine and terminate the process. Radical species are eventually stabilized into nonradical compounds. Natural muscle components, such as Fe, myoglobin (Mb), H<sub>2</sub>O<sub>2</sub>, and ascorbic acid, may favor lipid oxidation by exerting themselves by catalytic actions or by promoting the formation of ROS (Min and Ahn, 2005). Oxidative reactions can also be initiated by physical agents, such as irradiation and light ( $\approx$  photo-oxidation). Peroxides are commonly formed as primary lipid oxidation products and can subsequently undergo scission to form lower molecular weight volatile and non-volatile compounds (secondary oxidation products), such as carbonyls, alcohols, hydrocarbons, and furans (Min and Ahn, 2005). Among these, aldehydes are one of most abundant lipid oxidation products found in meat, with hexanal, malondialdehyde (MDA), and 4-hydroxy-2-*trans*-nonenal (4-HNE) being some remarkable examples (Ladikos and Lougovois, 1990). Detailed description of lipid oxidation mechanisms can be found in the classic (Ladikos and Lougovois, 1990) and updated review articles (Min and Ahn, 2005; Bekhit et al., 2013). The chemistry behind lipid

oxidation has been known for decades and described in detail. Conversely, the fact that food proteins could also be targets of ROS was a relatively recent discovery. The first reports on the occurrence of protein oxidation in muscle foods were published in the early 1990s and subsequently reviewed by Xiong (2000). Theoretically, the chemical and physical agents above described as potential initiators of lipid oxidation may also be able to initiate protein oxidation. As for lipid oxidation, protein oxidation is typically mediated by ROS and the general description of the protein oxidation reaction may also involve the phases of initiation, propagation, and termination (Xiong, 2000; Stadtman, 1992). Nevertheless, the reaction kinetics, the underlying mechanisms, and pathways as well as the oxidation products are fairly different (Stadtman, 1992). The peptide backbone and the functional groups located in the side chain of amino acid residues are common targets for ROS. The specific routes and chemical nature of the final protein oxidation products depend on the target, the oxidizing agent, and the intensity of the pro-oxidative conditions (Davies, 2005). The oxidative modification of the aminoacid side chains, the fragmentation of the peptide backbone, and the formation of intra- and inter-molecular cross-links (dityrosines, disulphide bonds), are common consequences of ROS mediated protein oxidation (Stadtman, 1992). The formation of protein carbonyls ( $\approx$  protein carbonylation) is one of the most remarkable consequences of oxidative damage to muscle proteins and involves the radical mediated metal-catalyzed oxidation of the side chains of alkaline aminoacids (lysine, arginine, and proline) (Estevez, 2011). The overall extent of protein carbonylation is a common measure of protein oxidation, while particular protein carbonyls, such as the  $\alpha$ -amino adipic semialdehyde (AAS), are used as specific markers of oxidative reactions in biological and food systems (Stadtman, 1992; Estevez, 2011). Besides ROS, reactive nitrogen species (RNS) may also be able to induce oxidative stress in muscle proteins (Skibsted, 2011).

Nitrite is as such, a pro-oxidant compound, which is able to abstract an electron from an oxidizable molecule (Honikel, 2008; Skibsted, 2011). The combination of nitrite and ascorbate, usually leads to antioxidant effects in muscle lipids via formation of nitric oxide that may act as an alkyl radical scavenger (Skibsted, 2011). A recent study in model systems reported nitrite had a negligible effect on protein oxidation, but induced the formation of a specific marker of nitrosative reactions, the 3-nitrotyrosine (Villaverde et al., 2014). A full understanding of the chemistry of RNS, the implication of such species in the oxidative and nitrosative damage to meat proteins, and the consequences of such chemical modifications on meat quality, requires further attention. As targets and sources of radical species, muscle lipids, and proteins may interact during the oxidative reactions albeit the precise mechanisms and pathways are still under study. Radical species may be transferred from lipids to proteins and viceversa, initiating the oxidation of neighbouring molecules (Lund et al., 2011). According to reaction constant rates between free radicals and biomolecules, certain ROS (such as the hydroxyl radical) would readily react with highly susceptible amino acid residues, such as methionine (Cadenas and Davies, 2000). The premature and preferential oxidation of these amino acids in biological systems could be regarded as a “protection” system, by which amino acid residues with antioxidant potential and irrelevant biological significance would scavenge ROS and hence, protect valuable residues and biomolecules (including unsaturated lipids) against oxidation (Estévez, 2015). As soon as the antioxidant capacity of proteins and of other redox-active compounds in the environment is exceeded, proteins, lipids and other susceptible molecules may undergo oxidative deterioration according to the chain reaction mechanisms previously described. The oxidation of other highly susceptible amino acids (such as tryptophan) may occur as an early event in protein oxidation and simultaneously to the oxidation of unsaturated fatty

acids (Viljanen et al., 2004; Estevez et al., 2008). However, once the oxidative reactions begin, the measurable changes indicate that lipid oxidation would progress faster than protein oxidation (Viljanen et al., 2004; Estevez et al., 2008). In a like manner, radicals and hydroperoxides formed from unsaturated lipids may attack susceptible amino acid side chains to yield carbonyl moieties. The formation of low molecular weight lipid oxidation products, such as thiobarbituric acid reactive substances (TBARs) and hexanal, may appear much sooner than late protein oxidation products, such as carbonyls and crosslinks (Viljanen et al., 2004; Estevez et al., 2008). It is worth noting that many of these oxidation products (particularly lipid and protein carbonyls) hold a higher activity and may be involved in further reactions. Lipid carbonyls, namely MDA and 4-HNE, may actually form complexes with food proteins via carbonylamine reactions (Zhao et al., 2012). Protein carbonyls, such as  $\alpha$ -amino adipic and  $\gamma$ -glutamic semialdehydes (AAS and GGS), may also be involved in advance reactions including Strecker-type and Schiff base formation (Estevez, 2011). For an in-depth knowledge of protein oxidation mechanisms and pathways, comprehensive reviews in medical research (Stadtman, 1992; Davies, 2005) and food science (Estevez, 2011; Lund et al., 2011) are available.

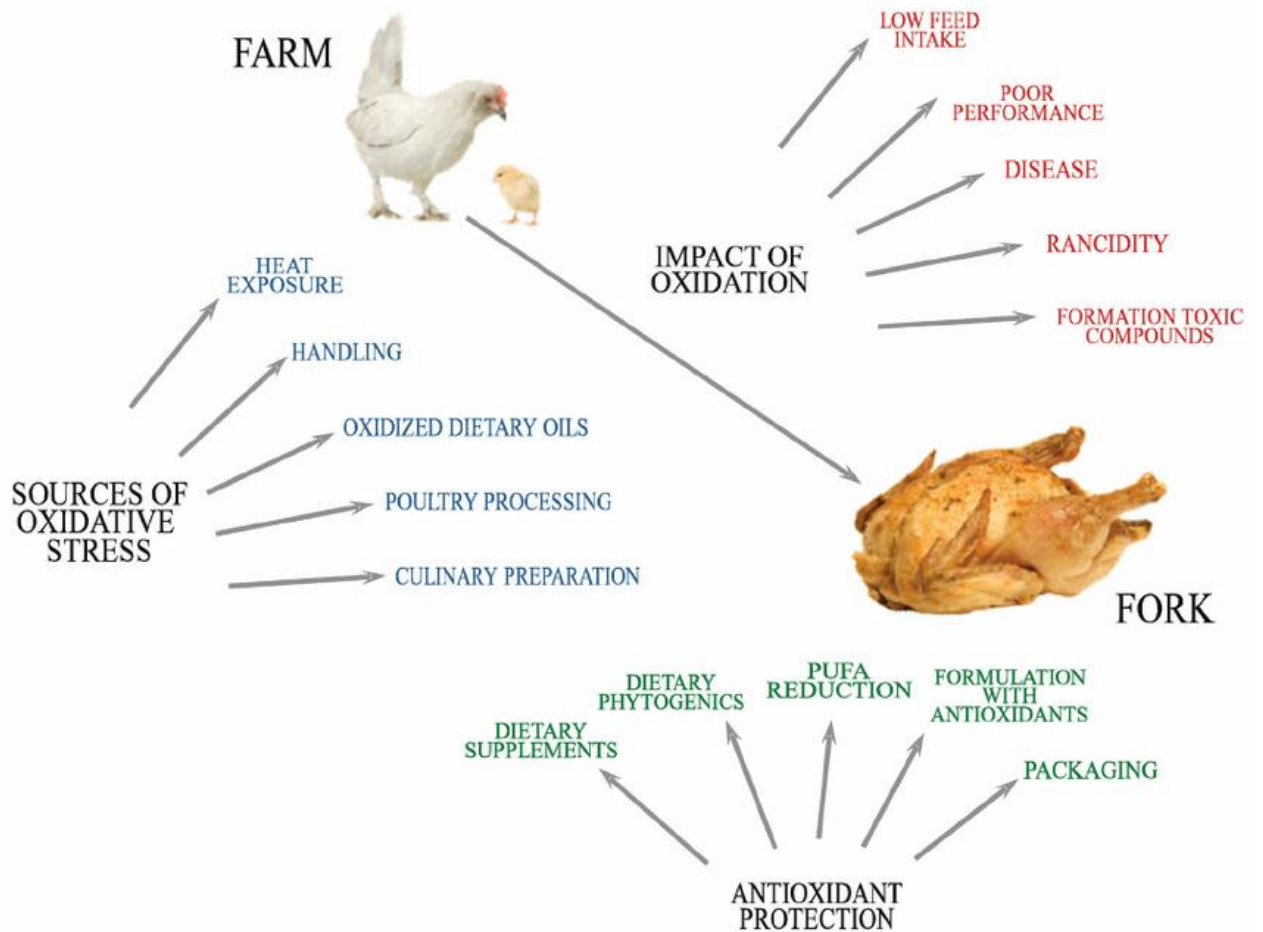


Figure 1. Oxidative damage to poultry: Sources of oxidative stress, impact of oxidation, and antioxidant strategies (Adapted from Estévez et al., 2015).

### 2.2.3 Lipid oxidation

Lipid oxidation is one of the primary mechanisms of quality deterioration in foods and especially in meat products. The changes in quality are manifested by adverse changes in flavor, color, texture and nutritive value, and the possible production of toxic compounds. The commonest type of oxidation during processing and storage of meat and meat products (and foodstuffs in general) is the autoxidation of fatty acids (Gandemer, 2002; Santos-Fandila et al., 2014). Only unsaturated fatty acids are oxidised by atmospheric oxygen at ordinary



temperatures. Saturated fatty acids (at tertiary carbons) enter the autoxidation process at higher temperatures associated with processing methods, such as boiling, baking, frying, roasting, etc. (Velíšek and Hajšlová 2009; Resconi et al., 2013). This is due to various degrees of bond dissociation energy at various positions in fatty acids.

Lipid oxidation in muscle systems is initiated at the membrane level in the intracellular phospholipid fractions. How this occurs has still not been resolved, even though it is generally believed that the presence of transition metals, notably iron, is pivotal in facilitating the generation of species capable of abstracting a proton from an unsaturated fatty acid.

#### 2.2.4 The mechanisms of lipid oxidation

Lipid oxidation takes place as a radical chain reaction characterized by three steps—initiation, propagation and termination (Falowo et al., 2014; Santos-Fandila et al., 2014) (Fig. 2).

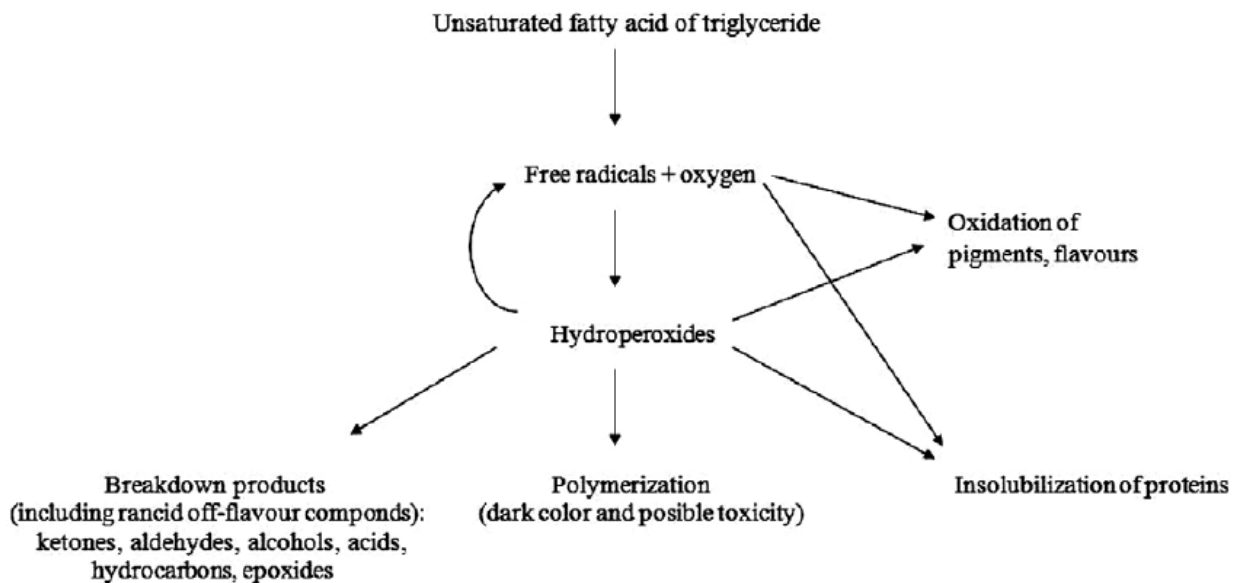


Figure 2: Mechanism of lipid oxidation (Adapted from Santos-Fandila et al., 2014).

- **Initiation**

During initiation, the homolytic cleavage of the covalent bond in the C-H hydrocarbon chain takes place with the formation of a free hydrogen radical (H•) and fatty acid free radical (R•) (1) (Velíšek and Hajšlová, 2009). Catalysts of this reaction include heating, radiation, metal ions and other free radicals (Hájek et al., 1998; Dave and Ghaly, 2011).



Cleavage also occurs by means of a reaction with another free radical or a reaction with metals with transitory valence (Velíšek and Hajšlová, 2009).

### • Propagation

A reaction occurs in the second step of oxidation between the highly reactive fatty acid radical and atmospheric oxygen to form a highly reactive peroxy radical (ROO•) (2), which is capable of reacting with unsaturated fatty acids to form hydroperoxides (ROOH) (3) (Wąsowicz et al., 2004; Velíšek and Hajšlová, 2009).



The sequence of two given reactions may be repeated a number of times, for which reason oxidation is known as a chain reaction (Velíšek and Hajšlová, 2009). The reaction of a fatty acid free radical with oxygen is far quicker than the reaction between a peroxy radical and a lipid hydrocarbon chain. The second reaction therefore determines the speed of autoxidation (Wąsowicz et al., 2004; Velíšek and Hajšlová, 2009).

### • Termination

In the final phase of oxidation, i.e. termination, non-radical relatively stable products are formed if the concentration of free radicals is sufficiently high (4, 5 and 6) (Wąsowicz et al., 2004; Velíšek and Hajšlová, 2009; Dave and Ghaly, 2011).



If the availability of oxygen is restricted, when the speed of oxidation depends on its partial pressure, the main radicals in the system are fatty acid radicals and the main termination reaction is their recombination (4). If sufficient oxygen is available, the reaction speed is not dependent on its partial pressure and more peroxy radicals are formed. The main reactions are then the recombination of fatty acid radicals with peroxy radicals and the mutual recombination of peroxy radicals (5, 6) (Velíšek and Hajšlová, 2009).

### **2.2.5 Protein oxidation**

The involvement of oxidized proteins to the development of biological diseases has been studied for a few decades, but the effects and the mechanisms of protein oxidation in food systems are largely unknown. Protein oxidation is defined as the covalent modification of a protein induced either by the direct reactions with reactive oxygen species (ROS) or indirect reactions with secondary by-products of oxidative stress. ROS can cause oxidation in both amino acid side chains and protein backbones, resulting in protein fragmentation or protein-protein cross-linkages. Although all amino acids can be modified by ROS, cysteine, and methionine that are the most susceptible to oxidative changes due to high reaction susceptibility of the sulfur group in those amino acids. Oxidative modifications of proteins can change their physical and

chemical properties, including conformation, structure, solubility, susceptibility to proteolysis, and enzyme activities. These modifications can be involved in the regulation of fresh meat quality and influence the processing properties of meat products. Oxidative stress occurs when the formation of oxidants exceeds the ability of antioxidant systems to remove ROS in organisms. Increased levels of protein oxidation have been associated with various biological consequences, including diseases and aging, in humans and other animal species.

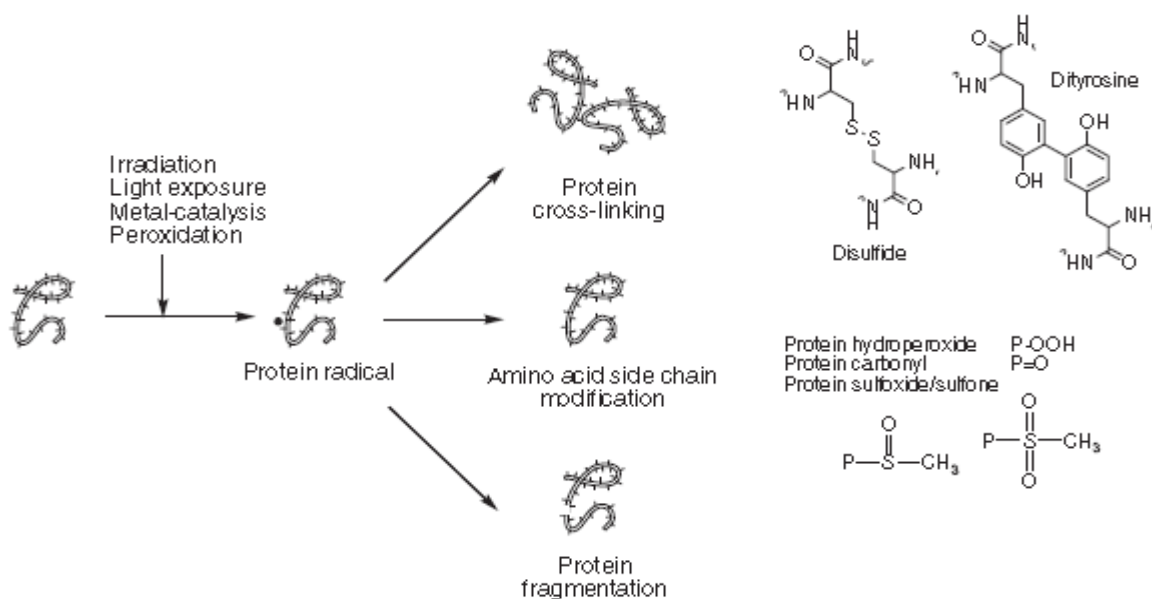


Figure 3: The most common consequences of oxidation of proteins (Lund et al., 2011)

The mechanisms of protein oxidation include amino acid side chain modification, protein cross-linking, and protein fragmentation (Lund et al., 2011). The oxidative stability of both dairy and meat proteins, as well as oxidative reactions of proteins isolated from various plant sources, has been studied. In these studies, versatile tools including both conventional spectrometric and fluorometric tools, as well as newly developed LC-MS methods, were applied. Lipid oxidation

products, such as hydroperoxides and carbonyls, are known to interact with amino acids, peptides and proteins. Recently, it was shown by using LC-MS tools that MDA does form protein-lipid adducts with peptides isolated from whey protein and lactalbumin (Gürbüz and Heinonen 2014). However, oxidation of food proteins is not only catalyzed by lipid oxidation products, but also proteins in fat-free food products or in foods with reduced fat content undergo oxidative changes. Depending on the amino acid composition, tryptic isolates of whey proteins produce methionine sulfoxide and sulfone, formylkynurenine, and dityrosine when oxidized in a metal catalyzed reaction in a non-lipid environment (Koivumäki et al 2012; Wang and Heinonen, 2016). Lysine oxidation in meat products, for example, may be monitored by following semialdehyde formation using LC-MS (Lund et al., 2011). Oxidation of essential amino acids (such as methionine, lysine, tyrosine and tryptophan) may be of concern during processing and storage. In addition to decrease in nutritional value, protein oxidation products may be of health concern such as formylkynurenine, which results from oxidation of tryptophan. Food proteins also have many functional properties (including stabilizing of emulsions, gels and foams, and water binding properties), which could be modified by oxidation.

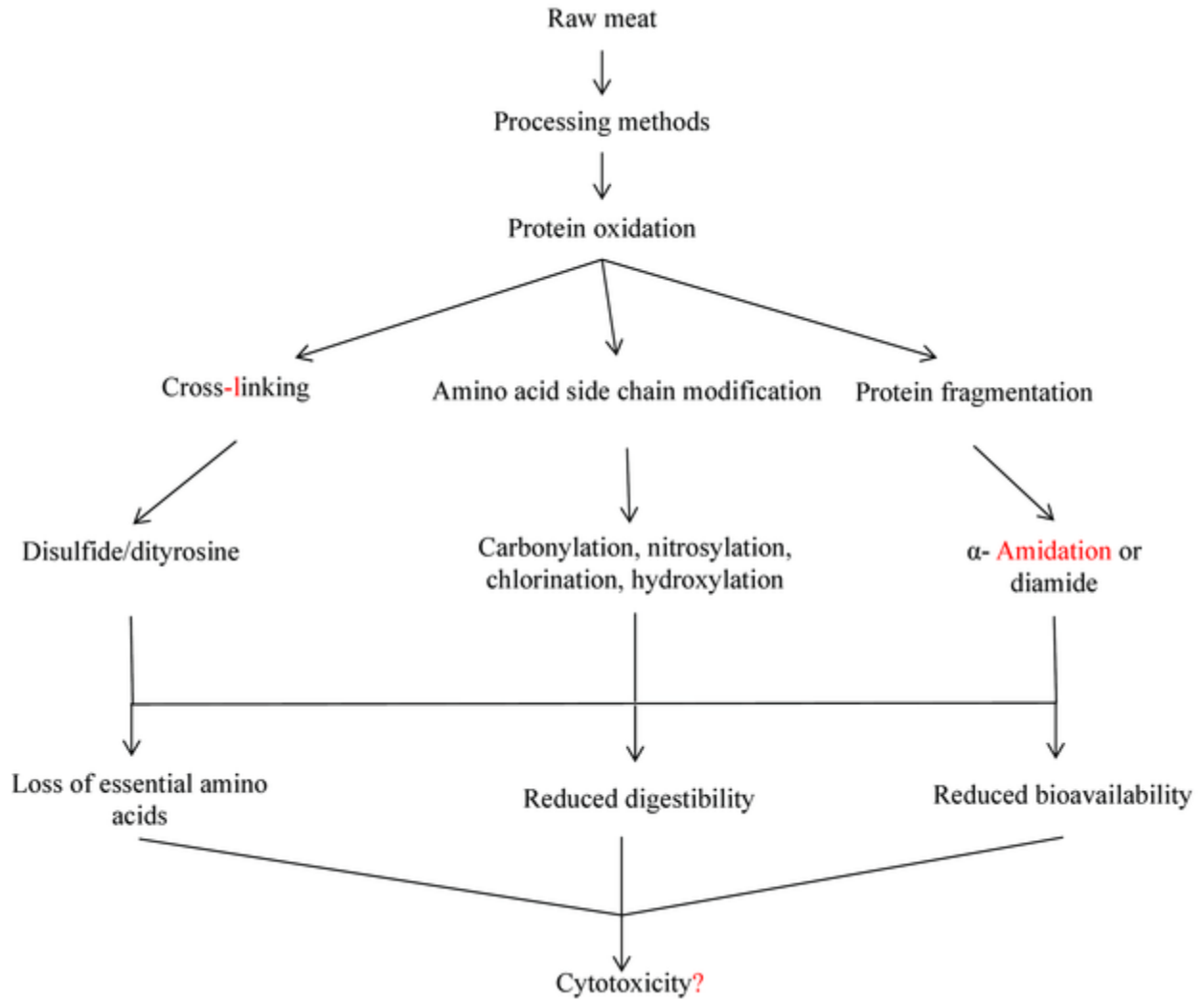


Figure 4: Proposed fate of protein during meat processing and possible implications on human health (Soladoye et al., 2015).

### 2.3 Antioxidant mediation of meat oxidation

Lipid and/or protein oxidation may be minimized by increasing the antioxidant concentration, utilizing modified atmosphere packaging or by the production of antioxidant compounds (e.g., Maillard reaction products) during processing.

### **2.3.1 Endogenous antioxidants**

There are a variety of endogenous antioxidant enzymes and compounds found in muscle and that serve to protect against *in vivo* oxidation (Decker and Mei, 1996). For example, enzymes include glutathione peroxidase, catalase, and superoxide dismutase (Decker and Xu, 1998). Antioxidant compounds include glutathione, carnosine and tocopherol. The two groups of dietary antioxidants that have received the most research attention to date are carotenoids and vitamin E. Representative compounds in each of these are naturally provided in forages or can be supplemented in feed concentrates. Carotenoids are liposoluble compounds that can accumulate in fat and produce colored fat in the carcasses of red meat species (Prache and Theriez, 1999; Dunne et al., 2009). In the United States, yellow fat is considered undesirable relative to white fat and is discriminated against. Additionally, carotenoids have not been demonstrated to provide consistently significant protection against lipid oxidation in red meat. The dietary supplementation of vitamin E (i.e.,  $\alpha$ -tocopheryl acetate) to meat producing animals has been consistently shown to increase  $\alpha$ -tocopherol concentrations in muscle and fat subsequently obtained from these animals (Faustman, 2004). This has led to decreased lipid oxidation in beef (Faustman et al., 1989), veal (Igene et al., 1976), pork (Buckley and Connolly, 1980, Guo et al., 2006), and lamb (Wulf et al., 1995; Strohecker et al., 1997). Faustman et al. (1999b) identified products of  $\alpha$ -tocopherol in meat consistent with the peroxy radical scavenging activity of this antioxidant. Interestingly,  $\alpha$ -tocopherol appears capable of increasing the redox stability of myoglobin in selected red meats (Faustman et al., 1989). Oxymyoglobin stability is improved by

*in vitro*  $\alpha$ -tocopherol (Yin et al., 1993) and in beef (Arnold et al., 1992) in a concentration-dependent manner. The redox stabilizing effects of elevated tissue concentrations of  $\alpha$ -tocopherol on bovine (Schaefer et al., 1995; Buckley et al., 1995; Faustman et al., 1998) and ovine myoglobins (Wulf et al., 1995; Guidera et al., 1995; Strohecker et al., 1997; Lauzurica et al., 2005) are well documented in the literature. However, unlike beef, a color-stabilizing effect of  $\alpha$ -tocopherol has not generally been observed in pork (Asghar et al., 1991; Hoving-Bolink et al., 1998; Cannon et al., 1996; Phillips et al., 2001a). One hypothesis for the basis of the protective effect of  $\alpha$ -tocopherol towards oxymyoglobin states that  $\alpha$ -tocopherol delays oxidation of lipid and subsequent release of secondary oxidation products, which are pro-oxidative towards oxymyoglobin (Faustman et al., 1998). This would represent an indirect effect of  $\alpha$ -tocopherol in maintaining acceptable beef color and is consistent with the known function of  $\alpha$ -tocopherol as a liposoluble antioxidant. The fatty acid profile of pork is more unsaturated than that of beef, and thus it would be expected to generate secondary products of lipid oxidation that could affect the redox stability of myoglobin. Recent reports suggest that species-specific differences in myoglobin primary structure (Suman et al., 2007) and sarcoplasm composition (Ramanathan et al., 2009) could explain the lack of an effect in pork. Arnold et al. (1993) established an effective concentration of 3.3 mg tocopherol/g beef muscle for minimizing lipid and myoglobin oxidation.

### **2.3.2 Exogenous antioxidants and processing**

Muscle-based foods contain a variety of antioxidant enzymes and metabolites that have the potential to mediate the oxidation process (Decker and Mei, 1996). Most attempts to reduce lipid and pigment oxidation in meats have focused on exogenous (i.e., ingredient) addition of pure synthetic and/or natural antioxidant molecules/mixtures, animal-derived proteins (Elias et al.,



2008; Wang et al., 2008) and antioxidant-containing plant materials. The active antioxidant components may function as free radical scavengers, reducing agents and/or chelators of catalytic metal ions.

Free radical quenchers and free radical scavengers delay or inhibit lipid oxidation by reacting with free radicals generated during the initiation or propagation steps. Synthetic phenolic antioxidants include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ). It is understood that the inclusion of BHA and BHT in ground beef patties led to significantly reduced lipid oxidation when compared to controls. BHA and BHT treatments had at least a two-fold lower lipid oxidation value when compared to untreated controls or other natural antioxidants (i.e., Fenugreek extracts) (Hettiarachchy et al., 1996). In contrast, the addition of BHA and BHT (100 mg/kg each) to a coating batter subsequently applied to ground pork patties that were deep fried did not result in antioxidant protection, when compared to control patties during 35 days of storage at 4 °C (Biswas et al., 2004). Combining antioxidant strategies can be especially effective. Many investigators have reported complementary effects (additive or synergistic) of antioxidants with different functional attributes (McBride et al., 2007). Ansorena and Astiasaran (2004) demonstrated that combining BHA and BHT with vacuum packaging of dry fermented pork sausages minimized lipid oxidation during storage at 4 °C for 5 months. Irradiated ground beef produced with BHA plus BHT with either ascorbate, erythorbate and trisodium phosphate individually or in combination exhibited significantly lower TBARs and greater redness values than control samples during 9 days of simulated retail display (Duong et al., 2008). The role of natural (cloves, cinnamon, ascorbic acid and Maillard reaction products (MRPs)) and synthetic (BHA, TBHQ and PG) antioxidants in controlling warmed-off flavor and non-haem iron release

in cooked and refrigerated stored meats from lamb, beef and pork was tested by Jayathilakan et al. (2007a). Their susceptibility to lipid oxidation was pork > beef > sheep, whereas the order of antioxidant activity for the natural antioxidants was MRPs > cloves > ascorbic acid > cinnamon; for synthetic antioxidants, it was TBHQ > BHA > PG.

$\alpha$ -Tocopherol is a natural radical-scavenging phenolic antioxidant that may be delivered through dietary approaches or ingredient addition. The latter provides the potential for a more cost-effective approach for incorporating  $\alpha$ -tocopherol into meat products. The use of  $\alpha$ -tocopherol as a processing ingredient to delay lipid oxidation and stabilize meat color has demonstrated inconsistent results. Aksu and Kaya (2005) used BHA and  $\alpha$ -tocopherol each at 50 and 100 mg/kg diced beef in the preparation of cooked and sliced kavurma. Improved color stability and decreased TBARs values in the products occurred in the following order: 100 mg BHA > 100 mg  $\alpha$ -tocopherol > 50 mg BHA > 50 mg  $\alpha$ -tocopherol > control, during storage under vacuum at 4 °C for 300 days. Crackel et al. (1988) reported that mixed tocopherols combined with ascorbyl palmitate and citric acid were as effective as TBHQ in retarding lipid oxidation in restructured beef steaks over 12 months frozen storage. Miles et al. (1986) found that  $\alpha$ -tocopherol effectively controlled oxidation of restructured pork during refrigerated storage at 4 °C for 16 days. Contrary to these studies, tocopherols at concentrations up to 1000 mg/kg meat had little effect on lipid oxidation in processed pork products stored at 18 °C for 37 weeks (Channon and Trout, 2002). A similar lack of antioxidant effect of  $\alpha$ -tocopherol was reported by Vara-Ubol and Bowers (2001), in which inclusion of the antioxidant (0.03% wt/wt) to ground, cooked pork failed to inhibit the formation of hexanal during six days storage at 4 °C. The same authors reported that sodium tripolyphosphate (STP; 0.2±0.3%) was more effective than  $\alpha$ -tocopherol; a combination of  $\alpha$ -tocopherol with 0.2% STP did yield enhanced antioxidant

activity relative to controls (no antioxidant) and tocopherol or STP alone. Directing the added antioxidant to or near the origin of oxidation sites is critical to efficacy and likely explains the basis for inconsistent results when tocopherols are added as ingredients (Wills et al., 2007). This was demonstrated convincingly by Mitsumoto et al. (1993), when they compared the antioxidant activity of  $\alpha$ -tocopherol delivered through dietary means and when added exogenously. These investigators achieved relatively high levels of  $\alpha$ -tocopherol in the *Longissimus* muscle of cattle through dietary supplementation and then compared the progression of lipid oxidation in ground beef of control cattle in which exogenous  $\alpha$ -tocopherol was added in an oil carrier to achieve an equivalent antioxidant concentration. The proper positioning of  $\alpha$ -tocopherol within the muscle tissues biomembranes by the living animal, a goal that could not be assured by the exogenous addition of  $\alpha$ -tocopherol as an ingredient. Raghavan and Hultin (2004) used a non-red meat (i.e., fish muscle) model to demonstrate the partitioning of added  $\alpha$ -tocopherol between neutral and polar lipid fractions as a function of the carrier used. Ethanol was more effective than canola oil in obtaining a greater relative concentration of antioxidant in the polar lipid fraction. Wills et al. (2007) subsequently hypothesized that vitamin E applied to muscle foods in an oil carrier would associate with the neutral lipid fraction (triacylglycerols) instead of the biomembrane's polar lipid fraction and be less effective. They demonstrated that delivery of  $\alpha$ -tocopherol using a polar carrier (i.e., ethanol) significantly reduced the TBARs values in cooked ground beef.

The addition of reducing agents (e.g., ascorbic acid, erythorbic acid and their salts), improves color stability and product storage life. Ascorbic acid at low levels (up to 250 ppm) catalyses the development of lipid oxidation, whereas at higher levels (500 ppm) is considered to inhibit the reaction, possibly by altering the relative concentration of ferrous and ferric iron, or by acting as oxygen scavenger (Sato and Hegarty, 1971; Igene et al., 1985). The ability of

ascorbic acid to delay lipid oxidation is attributed to its ability to scavenge oxygen, to regenerate the activity of primary antioxidants and to inactivate prooxidants (Bauernfeind and Pinkert, 1970; Bruno and Traber, 2006). The ingredient addition of ascorbic acid or ascorbate has led to reduced lipid oxidation (Shivas et al., 1984; Mitsumoto et al., 1991; Realini et al., 2004) and improved color stability (Greene et al., 1971; Mitsumoto et al., 1991; Realini et al., 2004) in beef. Hood (1975) demonstrated that injection of sodium ascorbate (500 mL, 50% w/v, pH 7.2) into cattle immediately prior to slaughter led to improved color stability of beef subsequently obtained from the treated animals. The reducing activity of ascorbic acid appears to improve muscle color stability via metmyoglobin reduction (Lee et al., 1999). Unlike vitamin E, the dietary supplementation of vitamin C has not demonstrated any antioxidant advantage in red meat subsequently obtained from treated animals (Gebert et al., 2006). Phillips et al. (2001b) reported that the addition of erythorbic acid to ground beef patties at 0.04% and 0.06% (w/w) concentrations resulted in prolonged red color in patties cooked to internal end point temperatures of 60, 66, 71 or 77 °C from the raw state; an undesirable consequence leading to premature browning (Killinger et al., 2000). The addition of sodium erythorbate, erythorbic acid, sodium ascorbate, ascorbic acid and ascorbyl palmitate to ground beef at a concentration of 2.3 mM to fresh ground beef significantly decreased lipid oxidation and increased the total reducing activity relative to controls during refrigerated and frozen storage (Sepe et al., 2005). Ascorbic acid and its salts and isomers have been combined with other functional antioxidants. An antioxidant combination containing radical quencher (0.2% w/w, rosemary extract), sequestrant (0.5% w/w, sodium citrate) and reductant (1000 ppm sodium erythorbate) incorporated into n-3 PUFA fortified fresh pork sausages significantly reduced TBARs and lipid hydroperoxides, and stabilized meat color during refrigerated (4 °C) and frozen (18 °C) storage (Lee et al., 2006). The

addition of vitamin C with lactic acid and clove oil as a dipping marinade for buffalo meat steaks did not result in any additional inhibition of lipid oxidation compared to lactic acid and clove oil alone (Naveena et al., 2006). However, vitamin C inclusion helped stabilizing buffalo meat color during storage. A mixture of rosemary, ascorbic acid, sodium lactate and red beet root extract significantly reduced lipid and pigment oxidation of fresh pork sausages during storage at 2 °C for 20 days (Martinez et al., 2006).

Metal chelators Phosphate is commonly injected to whole muscle processed red meats for its ability to improve water-holding capacity and cooked product yields. It also can act as a chelator to greatly decrease the catalytic activity of metal ions and potentially minimize lipid oxidation (Love and Pearson, 1971; Trout and Dale, 1990). Reports of no effect of phosphates on red meat oxidation have also been published (Akamittath et al., 1990; Stika et al., 2007). The addition of 2% EDTA was hypothesized to chelate non-haem iron released on cooking, which led to significant reductions in meat lipid oxidation (Igene et al., 1979). Experiments with ground pork demonstrated that the sequestering agents catechol, EDTA, diethylenetriamine pentaacetic acid, sodium pyrophosphate, and to a lesser extent sodium tripolyphosphate, lowered fat oxidation and improved sensory quality of stored cooked meat products (Shahidi et al., 1986). Darmadji and Izumimoto (1994) observed that the addition of chitosan (1%) led to a 70% decrease in TBARs values of meat after three days at 4 °C. The mechanism of inhibition was suggested to be related to chelation of free iron released from meat haemoproteins during heat processing or storage. The rate of lipid oxidation in fresh pork sausages was significantly decreased by addition of 0.5 to 1.0% chitosan, while samples containing both chitosan and nitrites (150 ppm) showed the lowest MDA values during 28 days storage at 4 °C (Soultois et al., 2008).

### **2.3.3 Natural source antioxidants**

The use of natural ingredients, especially of plant origin, in red meat products has increased in recent years. Current research suggests that most fruits, vegetables, nuts, cereals and spices contain antioxidant bioactive phytochemicals with the potential for minimizing oxidation in fresh and processed red meat products (Shahidi et al., 1992). The polyphenols are a class of phytonutrients with well-known health benefits. Significant polyphenols include tannins, flavonoids and anthocyanins (Balasundram et al., 2006). It is important to note, however, that it is difficult to compare the efficacy of different preparations of antioxidant-containing plant materials. Each plant (and its extracts) can contain a large variety of bioactive compounds with antioxidant activity. In order to compare the relative effectiveness of different plant materials, the relevant constituent antioxidants must be standardized in some manner. Differences in cultivars, geography and climate, soil conditions and many other factors make such comparisons very challenging. Dietary fibres with significant antioxidant activity from citrus fruits (Fernandez-Lopez et al., 2004), wheat bran (Vitaglione et al., 2008), oat bran (Persson et al., 2004) and rice bran (Choi et al., 2009), have been incorporated in the formulations of meat products including ground meat and sausages. Buffalo meat loaves, breakfast sausages, fermented sausages, restructured beef steaks and beef patties have been prepared with natural antioxidants from plant-derived ingredients, such as carrots (Devatkal et al., 2004), onion and leek (Fista et al., 2004), tomato peel and seeds (Calvo et al., 2008) and cabbage (Chu et al., 2002). Antioxidant activity has been reported for walnut components (Serrano et al., 2006), grape seed and bearberry extract in raw and cooked pork (O'Grady et al., 2008), plum concentrates and powder in precooked roast beef (Nunez de Gonzalez et al., 2008), cherry and

apple fruits in ground beef patties (Britt et al., 1998), and green tea extract in fresh pork sausages (Valencia et al., 2008). In a comprehensive work, McCarthy et al. (2001) reported the antioxidant activities of aloe vera, fenugreek, mustard, tea catechins and ginseng in cooked pork patties. Tea catechins are potent natural antioxidants and have exhibited greater antioxidant efficacy compared to vitamin C in cooked or raw beef (Mitsumoto et al., 2005). Han and Rhee (2005) investigated antioxidant properties of white peony, red peony, sappanwood, Mountain peony, rehmania, and angelica in ground, raw and cooked chevon and beef during refrigerated storage. Significant reductions in lipid oxidation and discoloration were recorded in ground chevon and beef patties, respectively. Hernandez-Hernandez et al. (2009) evaluated the antioxidant effects of rosemary and oregano extracts on TBARs and color of model raw pork batters. They observed greater antioxidant activity for rosemary extracts compared to oregano ones and attributed the higher antioxidant effect of rosemary to the presence of high concentrations of carnosic acid and carnosol, and unidentified active compounds. They further reported that oregano extracts containing high concentrations of phenols, mainly rosmarinic acid, efficiently prevented color deterioration. Lemon and orange extracts were reported to exert antioxidant effects in beef meat balls (Fernandez-Lopez et al., 2004), and pine bark extracts were found to be suitable antioxidants in food systems (Vuorela et al., 2005). The antioxidant effect of rosemary, marjoram, caraway, sage, basil, thyme, ginger and clove were reported in fresh pork sausages, raw and cooked minced meat patties and raw buffalo meat steaks (Abd El-Alim et al., 1999; Naveena et al., 2006; Georgantelis et al., 2007). Antioxidant activity of 10 spices (allspice, black pepper, cardamom, cinnamon, clove, coriander, cumin, ginger, nutmeg and rose petals) commonly used in the formulation of fermented meat sausages were evaluated for their antioxidative properties. Clove followed by rose petals and allspice were found to exhibit the

greatest antioxidant index when used in a dry form (Al-Jalay et al., 1987). There have been many reports of the antioxidant effectiveness of rosemary (*Rosmarinus officinalis L.*) extracts in red meat products. The antioxidant activity of rosemary extracts has been associated with the presence of carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol, which act primarily as radical scavengers (Basaga et al., 1997; Zheng and Wang, 2001). Effective concentration ranges of 500-1000 mg rosemary/kg in beef steaks (Stoick et al., 1991), 200-1000 mg/kg in various foods (Shahidi et al., 1992) and 1000 mg/kg in precooked-frozen sausage (Sebranek et al., 2005), have been reported. Rosemary powder alone (1000 ppm) and rosemary with ascorbic acid (500 ppm) incorporated within ground beef patties showed decreased metmyoglobin formation and lipid oxidation relative to ascorbic acid (500 ppm), taurine (50 mM), carnosine (50 mM), or their combinations (Sanchez-Escalante et al., 2001). Djenane et al. (2002) showed that when used in combination with vitamin C (500 ppm), rosemary (1000 ppm) was more effective in delaying myoglobin oxidation and lipid oxidation when sprayed on beef steak surfaces than combinations of taurine (50 mM) and vitamin E (100 ppm). Balentine et al. (2006) evaluated the timing of application of rosemary extract and concluded that optimal redness values, oxymyoglobin content and low TBARs values were achieved when rosemary was added as a pre-grinding treatment to beef. Maillard reaction products have been reported to also exert antioxidant activity (Manzocco et al., 2001; Benjakul et al., 2005). These can be formed *in vitro* and subsequently applied to meats (Jayathilakan et al., 1997) and are effective antioxidants (Jayathilakan et al., 2007a, b).

#### **2.4 Healthier meat and meat product formulations**



Most recent studies have addressed strategies to improve the safety of meat products while maintaining good sensory quality attributes either by combining high pressure with other technologies or by modifying product formulations. “Food additives” is a general term referring to anything added to food to achieve specific functions, for example, to aid in food processing, to increase the product’s nutritive value, to enhance the product’s palatability through regulating its physicochemical properties, and to extend the product’s storage stability. In muscle food processing, both synthetic and natural, generally recognized as safe (GRAS) chemical compounds and ingredients are used as functional non-meat additives to assure quality and safety of finished products. The main ingredients in a typical meat product are raw muscle and fat. Non-meat ingredients include a variety of additives that are small molecules (salt, phosphate, antioxidant, and antimicrobial compounds present in plant-derived spices and seasoning, etc.) or large molecules (starch, gums, non-muscle proteins, etc.). All these additives can be referred to as chemical ingredients. Non-muscle proteins and protein hydrolysates (peptides), such as soy protein, soy protein hydrolysate and sodium caseinate, are used in meat processing to improve textural characteristics and water-binding capacity in finished products. Common small ingredients influence meat product quality through interaction with proteins and lipids in meat processing. Examples include monobasic salts (NaCl, KCl, etc.), divalent cationic salts (CaCl<sub>2</sub>, MgCl<sub>2</sub>, etc.), various alkali and acid compounds, and different phosphates. Lipid and non-lipid free radicals, which are commonly generated in meat processing, can also have a profound impact on the quality of meats because they not only cause off-flavors but also impair products’ texture by reducing muscle protein functionality; for this reason, antioxidant compounds are widely used, to control and minimize oxidation.

### **2.4.1 Meat Product Reformulation Processes**

Technological strategies used to design and develop functional foods based on changes in meat transformation systems are especially promising. A number of approaches can be used to remove, reduce, increase, add and/or replace different bioactive components. Modification of meat formulation process also makes it possible to use traditional ingredients and other ingredients specifically designed with certain attributes that confer health-promoting properties.

### **2.4.2 Improving Fat Content and Replacement of Animal Fat with Vegetable Oils**

Three main goals have been identified for altering the fat content using meat reformulation strategies: reduction of total fat and energy, reduction of cholesterol, and modification of fatty acid profiles. Fat reduction is usually based on two main criteria, which involve either the utilization of leaner meat raw materials and/or the reduction of fat by adding water and other ingredients like carbohydrate based fat replacers or gums or protein based fat replacers with little or no calorific content. Replacement of fat already present in the meat with high MUFA and/or PUFA oils from vegetable sources (like olive, cottonseed, corn, soybean, peanut, etc.) and marine lipids, had been done by many researchers. Lee et al. (2006) prepared n-3 PUFA fortified fresh turkey and pork sausages by adding an emulsion containing 25% algal oil into the sausage batter. Marchetti et al. (2014) incorporated fish oil in low-fat sausage and found them to be acceptable. Low-fat meat products had been formulated by using fat substitutes like konjac gel (Osburn and Keeton, 1994), agar, curdlan gum and k-carrageenan (Hsu and Chung 2000).

Fat profile has also been modified by adding conjugated linolenic acid (CLA), which has antioxidant, anti-carcinogenic, immunomodulative and anti-obesity properties and helps in regulating bone metabolism and reduces the risk of diabetes (Bernardini et al., 2011).

Commercially produced CLA isomers have been injected into whole muscles (Baublits et al., 2007) and incorporated into meat products, such as sausages (Juarez et al., 2009), to achieve enough levels of CLA to produce health benefits when consumed in smaller portions.

Consumers are increasingly preferring products with low-fat content while retaining good flavor and overall acceptability (Lin & Huang, 2008). The increasing demand for low-fat diets has led the food industry to develop or modify traditional food products to contain less animal fat (Bloukas & Paneras, 1993; Choi et al., 2009; Garcia et al., 2002; Mittal & Barbut, 1994). An option for meeting both health and taste issues would be replacing animal fat with vegetable oils. Vegetable oils are free of cholesterol and have a higher ratio of unsaturated fatty acids (UFA) to SFA than animal fats (Liu et al., 1991); however, the use of vegetable oils does not seem technologically suitable because of their different physical properties (such as color and flavor), and more unsaturated fatty acid composition that make them prone to oxidation (Pappa et al., 2000). Therefore, hydrogenation and interesterification procedures have been successfully developed to modify the physical and chemical properties of vegetable oil (Liu et al., 1991; Vural et al., 2004). The food industry adopted the use of partially hydrogenated vegetable oils (PHVOs) to achieve the same functional characteristics provided by animal fats in food products (Eckel et al., 2007). Vural and Javidipour (2002) indicated that the replacement of beef fat with partially interesterified vegetable oils in frankfurters could improve the nutrient quality due to modification of the fatty acid composition. Liu et al. (1991) noted that vegetable oils in ground beef patties could reduce caloric and cholesterol content without detrimentally affecting the palatability of the product. Studies have shown the importance of unsaturated fatty acids, especially linoleic acid (n-3) and linolenic acid (n-6). Although PHVOs and the *trans*-FA (TFA) components produced by partial hydrogenation of liquid vegetable oils appeared to be a suitable

alternative from the stability and sensory standpoints, TFA have been associated with the development of CVD, so this approach has been abandoned.

Alternative oils with increased amounts of oleic acid (i.e., ~50-65%), decreased amounts of linoleic acid (i.e., ~20–30%), and low amounts of  $\alpha$ -linolenic acid ( $\sim <3\%$ ) have proven to be effective replacements for PHVOs, because they are able to withstand higher temperatures. Examples of high-oleic (HO) oils (i.e.,  $>70\%$  oleic acid) include olive oil, HO sunflower oil, HO safflower oil, and HO canola oil. Several papers indicate olive, palm, canola, linseed (Jiménez-Colmenero et al., 2001; Fernández-Ginés et al., 2005), soybean and cottonseed oils (Ambrosiadis et al., 1996), as appropriate vegetable sources for meat industry. A systematic review of HO vegetable oil substitutions for other fats and oils on cardiovascular disease risk factors suggested that replacing fats and oils high in SFA (such as palm oil) or TFA with either HO soybean oil or oils high in  $n-6$  PUFAs would have favorable and comparable effects on plasma lipid risk factors and overall CHD risk (Choi et al., 2009). Choi et al. (2010a) studied the effects of replacing pork back fat with vegetable oils and rice bran fiber on the quality of reduced-fat frankfurters. Ten percent of the total fat content of frankfurters with a total fat content of 30% (control) was partially replaced by one of the vegetable oils to reduce the pork fat content by 10%. The moisture and ash content of low-fat frankfurters with vegetable oil and rice bran fiber were all higher than the control ( $p < 0.05$ ). Low-fat frankfurters had reduced-fat content, energy values, cholesterol and TFA levels, and increased pH, cooking yield and TBARs values compared to the controls ( $p < 0.05$ ). Low fat frankfurters with reduced-fat content plus rice bran fiber had sensory properties similar to control frankfurters containing pork fat.

Tudose et al. (2014) investigated the effect of animal fat replacement with vegetable oils on sensorial perception of meat emulsified products and they showed that the newly obtained meat

product was well perceived by tasters. It seems that, during storage, aroma and taste positively evolved and intensified in the product formulated with the vegetable oil pre-emulsion. However, the recorded decrease in moisture led to an increase in firmness, especially for the new product.

The effects of reducing pork fat levels from 30% to 20% and partially substituting the pork fat with a mix of grape seed oil (0%, 5%, 10% and 15%) and 2% rice bran fiber in reduced-fat meat batters, were investigated based on chemical composition, cooking characteristics, physico-chemical and textural properties, and viscosity (Choi et al., 2010b). The incorporation of grape seed oil and rice bran fiber successfully reduced the animal fat content in the final products, while improving other characteristics.

Baek et al., (2016) investigated the effects of canola and flaxseed oils on the physico-chemical properties and sensory quality of emulsion-type sausage made from spent layer meat. Three types of sausages were manufactured with different fat sources: 20% pork back fat (CON), 20% canola oil (CA) and 20% flaxseed oil (FL). The results show that using canola or flaxseed oils as a pork fat replacer increased n-3 PUFA and lower SFA and the n-6/n-3 ratio compared to CON, in particular, canola oil resulted in a great emulsion stability and sensory quality.

Rodríguez-Carpena et al. (2011) demonstrated that partial replacement of back-fat (50%) by avocado (A), sunflower (S), and olive (O) oils caused a significant reduction of SFA and a concomitant enrichment in UFA. In contrast, the usage of vegetable oils as back-fat replacers had no impact on the formation of protein carbonyls.

Recent research conducted to improve UK-style sausages by incorporating rapeseed and sunflower oils as pre-formed emulsions with a total fat content of about 12%, as an alternative to pork backfat emulsion, reduced total SFA composition from 38% to 14%, increased MUFA composition from 45% to 59% and increased PUFA composition from 15% to 25% (Asuming-

Bediako et al., 2014). Eating quality, liking, color, shelf-life and lipid oxidation was not affected other than slight differences in the attributes 'firmness' and 'particle size'.

Youssef and Barbut (2011) concluded that substituting beef fat with canola oil or pre-emulsified canola oil (using soy protein isolate, sodium caseinate or whey protein isolate) in showed a positive effect on improving yield and restoring textural parameters.

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### 3. Objectives

The general objective of this PhD thesis was to study the effects of dietary supplementation, product formulation and storage conditions on the oxidative stability of lipids and proteins in meat and meat products, with the final aim to better understand the trends of these oxidative processes in such products in order to identify strategies to improve their overall quality.

The thesis work was divided in three subsections, with diverse specific objectives:

- 1) To determine the effects of light exposure on photooxidation on the oxidation of both lipid and protein fractions of raw beef and chicken breast meats, under commercial retail conditions.
- 2) To investigate the effectiveness of dietary plant extract mixture (*Ocimum sanctum*, *Embllica officinalis*, *Withania somnifera* and *Mangifera indica*) on the oxidative stability of chicken breast meat during chill and frozen storage.
- 3) To study the influence of partial or total replacement of pork back fat by hempseed oil emulsion on the oxidative stability and sensory characteristics of cooked sausages, with particular emphasis on both lipid and protein fractions.

## **4. Results**

4.1 Photooxidation of lipids and proteins of raw chicken and beef meats during storage under commercial retail conditions

4.2 Effect of plant extracts mixture on the oxidative stability of chicken breast meat during chill and frozen storage

4.3 Influence of pork back fat replacement by hempseed oil emulsion on the oxidative stability of lipids and proteins in cooked sausages

#### **4.1 Photoxidation of lipids and proteins of raw chicken and beef meats during storage under commercial retail conditions**

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## **Abstract**

The aim of this study was to determine the influence of photooxidation in both lipid and protein fractions of raw meat stored under commercial retail conditions. Beef and chicken meat slices were packed in vessels with transparent shrink film and exposed to white fluorescent light 4 °C for 0, 3 and 5 days (12-h light/12-h darkness cycle). Lipid oxidation was assessed by measuring peroxide value (PV) and thiobarbituric acid-reactive substances (TBARs), while the quantification of thiol and carbonyl groups were used to evaluate the extent of protein oxidation. In general, both lipid and protein oxidation parameters significantly increased during storage at 4°C under darkness conditions, with a greater impact when exposed to light. Beef meat exhibited much higher lipid and protein oxidation (about 12 and 2 times, respectively) than chicken breast. This study evidences that storage time and exposure to light can greatly affect both lipid and protein oxidations in raw meats, especially beef meat, thus suitable strategies (i.e. film types and/or active packaging) should be found to improve their oxidative stability and reduce the impact of light exposure.

*Keywords:* Photooxidation; Lipid oxidation; Protein oxidation; Chicken meat; Beef meat; Fatty acid composition; shelf-life



## **1. Introduction**

The consumption of meat and meat products is increasing worldwide, due to their rich nutritional composition (Dave & Ghaly, 2011), which consist of water, proteins, minerals, vitamins and fat; in fact, it is the main source of animal protein for human consumption (Hathwar et al., 2012; Santos et al., 2013; Beiloune et al., 2014). However, recent studies have established a likely relationship between red meat consumption and an increased risk of suffering serious health disorders, such as colorectal cancer and coronary-heart diseases (Ferguson, 2010). Amongst other factors, animal fat and particularly saturated fatty acids (SFA), have been recognized as influential factors of certain chronic diseases associated to meat consumption, such as heart failure, obesity and type 2 diabetes (McAfee et al., 2010; Corpet et al., 2011; McNeill & Van Elswyk, 2012; Wyness et al., 2011; Pan et al., 2012; Ferguson, 2010; Rodríguez-Carpena et al., 2012). Oxidation is a general process, which affects lipids, pigments, proteins, DNA, carbohydrates, and vitamins (Hawkins et al., 2009). Oxidation of lipids and proteins is in fact the main, non-microbial cause of meat quality deterioration during processing, which could be ascribed to an imbalance of the defense mechanism of the animal against oxidative stress as a result of the rapid depletion of endogenous antioxidants after slaughtering (Xiao et al., 2011). In muscle and fat tissue, the oxidative process continues postmortem and will affect the shelf-life of meat and meat products (Smet et al., 2008). The susceptibility of meat to undergo oxidative reactions involves many endogenous (i.e., fatty acid composition, heme iron content, enzymatic and non-enzymatic antioxidants) and external factors (i.e. pre-slaughter and slaughtering handling, aging, processing and storage conditions) (Min and Ahn, 2005; Lund et al., 2011). Low-temperature storage is one of the primary preservation methods to maintain meat freshness, because the rates of microbiological, chemical and biochemical changes are reduced. Post-

mortem refrigerated storage of meat, often termed as maturation or ageing, permits desirable degradative structural changes in myofibrillar and connective tissue proteins which enhance its palatability (Maqsood et al., 2015). Meat carcasses are held in refrigerated storage for varying periods to improve tenderness and to promote the formation of aroma compounds or their precursors that develop during cooking (Rodriguez-Estrada et al., 1997; Boselli et al., 2009; Cardenia et al., 2015). During the retail display, the product is frequently packed with transparent films, which favor their exposure to both light and oxygen (Dalsgaard et al., 2010; Cardenia et al., 2015). Oxidation of lipids and proteins can affect meat quality, and represents a leading cause for health concerns, consumer rejection, and economic losses (Xiao et al., 2011; Estevez, 2011; Lund et al., 2011; Bekhit et al., 2013). In particular, lipid oxidation represents a serious problem for meat industry, since it lowers the nutritional quality, limits the shelf-life, affects the sensory properties, and favors the development of potentially toxic compounds during processing and storage of meat and meat products (Sample, 2013). On the other hand, protein oxidation may result in modifications of the functional (ie. tenderness, water-holding capacity, etc) and nutritional properties of proteins, due to the formation of protein polymers, loss of solubility, changes in amino acid composition and increase of proteolytic susceptibility (Xiong, 2000). While lipid oxidation has been extensively studied in meat and meat products, the influence and the mechanisms of protein oxidation in these food products are still largely unknown. However, there is an increasing evidence on the occurrence of actual interactions between protein and lipid oxidation (Faustman et al., 2010), which seem to be time-dependent. The belief that lipid and protein oxidation are governed by similar chemical mechanisms explains that most strategies aimed to control protein oxidation in meat and meat products are those reported to be effective against lipid oxidation (Estévez, 2011). Proteins and lipids can break down resulting in the

production of new compounds causing changes in meat flavor, tenderness, juiciness, odour and texture (Dave & Ghaly, 2011). There are several methods to monitor lipid and protein oxidation in meat and meat products; peroxide value (PV) and thiobarbituric acid-reactive substances (TBARs) are frequently used as markers of primary and secondary lipid oxidation respectively, while the content of carbonyl and sulfhydryl groups are often employed to evaluate protein oxidative changes.

The extent of lipid oxidation and protein carbonylation in post-mortem meat is highly dependent on the origin of the meat, type of muscle, species, and the storage conditions (Estevez, 2011). The occurrence of early postmortem protein oxidation was described in beef muscles during aging/chill storage (Lindahl et al., 2010; Rowe et al., 2004), while the occurrence of early post-mortem lipid and protein oxidation has been found during aging/chill storage of chicken (Zhang et al., 2011). Poultry meat is rich in polyunsaturated fatty acids (PUFA) and is low in natural antioxidants (Barroeta, 2007; Grashorn, 2007), while beef meat is characterized by a high content of heme pigments (mainly myoglobin and hemoglobin) (Boselli et al., 2012). These composition traits make both types of meat quite susceptible to lipid oxidation during storage, especially if exposed to light, which could thus lead to the reduction of their nutritive value and accumulation of lipid oxidation products. To the best of our knowledge, no study has been carried out on lipid and protein photooxidation of raw beef and chicken meats during storage. The aim of this study was to determine the effects of light exposure on the oxidation of both lipid and protein fractions of raw beef and chicken meats, during storage under commercial retail conditions.

## **2. Materials and methods**

## 2.1 Reagents and solvents

All chemicals used were of analytical grade. Ammonium thiocyanate ( $\text{NH}_4\text{SCN}$ ,  $\geq 97.5\%$ ), barium chloride dihydrate ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), iron (II) sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), sodium dihydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), tri-sodium phosphate ( $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and double distilled water, were supplied by Carlo Erba (Milan, Italy). Chloroform, methanol, ethanol, hydrochloric acid ( $\text{HCl}$ , 37%), potassium chloride ( $\text{KCl}$ ), *n*-hexane and isopropanol, were purchased from Merck (Darmstadt, Germany). The standard mixture of fatty acid methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN, USA). 1,1,3,3-tetraethoxypropane (precursor for malondialdehyde (MDA)), trichloroacetic acid (TCA,  $\text{CCl}_3\text{COOH}$ ,  $\geq 99.0\%$ ), 2-thiobarbituric acid ( $\geq 98.0\%$ ), anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ,  $\geq 99.0\%$ ), sodium pyrophosphate ( $\text{Na}_4\text{O}_7\text{P}_2$   $\geq 95.0\%$ ), trizma maleate ( $\geq 99.5\%$ ), magnesium chloride ( $\text{MgCl}_2$ ,  $\geq 98.0\%$ ), ethylene glycol-bis(2-aminoethylether)-tetraacetic acid (EGTA  $\geq 97.0\%$ ), 2,4-dinitrophenylhydrazine (DNPH,  $\geq 97.0\%$ ), bovine album serum (BSA,  $\geq 96\%$ ), guanidine hydrochloride ( $\geq 99.0\%$ ), potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ,  $\geq 99.0\%$ ), ethyl acetate ( $\geq 99.7\%$ ), tris(hydroxymethyl)-aminomethane (TRIS,  $\geq 99.8\%$ ), sodium dodecyl sulfate (SDS,  $\geq 99.0\%$ ), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB,  $\geq 98.0\%$ ) and L-cysteine ( $\geq 98.5\%$ ), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Filters papers (90 and 185 mm diameter) were used (Whatman, Maidstone, England).

## 2.2 Sampling, packaging and experimental design

Raw chicken breast and beef meats (*Longissimus dorsi* (LD)) from three different animals, were purchased from a local supermarket (Bologna, Italy). Five slices of 80-100 g and 0.8-1 cm thickness were obtained from each type of meat ( $n= 15 +15$ ). Each slice was packed in a

polyethylene tray, which was wrapped with a transparent shrink film (14  $\mu\text{m}$  thickness) with 10,445  $\text{mL}/\text{m}^2/24$  h of oxygen permeability. The packed slices of each type of meat were subjected to the following storage conditions:

- (a) Three trays were immediately frozen ( $-20$   $^{\circ}\text{C}$ ), which represented T0;
- (b) Three trays were stored in the dark at  $4$   $^{\circ}\text{C}$  for 3 and 5 days in a bench refrigerator which represented T3D and T5D, respectively;
- (c) Three trays were stored at  $4$   $^{\circ}\text{C}$  for 3 and 5 days under a daylight lamp for 12 h per day in a bench refrigerator which represented T3L and T5L, respectively.

The daylight lamp had a temperature and power of 3800 K, 1200 lx and 36 W (Osram, Milan, Italy), respectively. The lamps were located 1.5 m above the samples. Once the meat samples had reached the specified storage time, they were stored at  $-20$   $^{\circ}\text{C}$  until analysis.

### **3. Analytical procedures**

#### *3.1 Lipid extraction*

Lipids were extracted according to Boselli et al. (2005), a modified version of the method suggested by Folch, Lees, and Sloane-Stanley (1957). The frozen samples were minced, a subsample of 15 g was taken, and homogenized with 200 mL of a chloroform:methanol solution (1:1, v/v) in a glass bottle with screw-cap. The bottle was kept in an oven at  $60$   $^{\circ}\text{C}$  for 20 min before adding 100 mL chloroform. After 3 min of homogenization, the mixture was filtered through filter paper; the filtrate was mixed thoroughly with 100 mL of a 1 M KCl solution and left overnight at  $4$   $^{\circ}\text{C}$  to allow phase separation. The lipid-containing phase (lower) was collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Two lipid extractions were performed per each sample.

### *3.2 Fatty acid composition*

About 20 mg of lipid extract were methylated with 200  $\mu$ L of diazomethane (Fieser & Fieser, 1967); 1.11 mg of tridecanoic acid methyl ester was added (as internal standard), and the mixture was transmethylated with 40  $\mu$ L of 2 N KOH in methanol (European Commission, 2002), vortexed for 1 min, kept for 5 min, and centrifuged at 1620  $\times$  g for 5 min. Supernatant was transferred to a vial before being injected into a gas chromatograph coupled to a flame ionization detector (GC-FID). The GC-FID instrument was a GC8000 series (Fisons Instruments, Milan, Italy) coupled to a computerized system for data acquisition (Chromcard Data System, ver. 2.3.1, Fisons Instruments). A Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropyl-polysiloxane) fused-silica column (105 m  $\times$  0.25 mm  $\times$  0.2  $\mu$ m film thickness) (Bellefonte, USA), was used. Oven temperature was programmed from 100  $^{\circ}$ C to 180  $^{\circ}$ C at a rate of 3  $^{\circ}$ C/min, kept at 180  $^{\circ}$ C for 10 min, and then taken to 240  $^{\circ}$ C at a rate of 3  $^{\circ}$ C/min; the final temperature was kept for 30 min. The injector and detector temperatures were both set at 250  $^{\circ}$ C. Helium was used as carrier gas at a constant pressure of 260 kPa. The split ratio was 1:50. Peak identification was carried out by comparing the peak retention times with those of the GLC 463 FAME standard mixture. The GC response factor of each fatty acid was calculated by using the GLC 463 FAME standard mixture and the internal standard (13:0). The limit of detection (LOD) of FAMES was 0.002 mg, whereas the limit of quantification (LOQ) was 0.006 mg. LOD and LOQ were calculated as signal-to-noise ratios equal to 3:1 and 10:1, respectively. Three replicates were analyzed per sample.

### *3.3 Peroxide Value (PV)*

Peroxide value was determined using a modified version of the method of Shantha and Decker (1994). In this method, peroxides oxidize ferrous ions to ferric ions, which react with ammonium thiocyanate to give rise to a colored complex that can be measured spectrophotometrically. Briefly, 20 mg of extracted lipids were mixed with 9.8 mL of chloroform:methanol (2:1, v/v) and 50  $\mu\text{L}$  of thiocyanate/ $\text{Fe}^{2+}$  solution, and then vortexed. After 5 min, the absorbance was measured at 500 nm using a double beam UV-VIS spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan). PV was calculated using a Fe (III) standard calibration curve with a concentration range of 0.599-47.95  $\mu\text{g}/\text{mL}$  ( $y = 0.028 + 0.019x$ ;  $r^2 = 0.996$ ). PV was expressed as meq of  $\text{O}_2$  per kg of fat. Two replicates were run per sample.

#### *3.4 Thiobarbituric acid reactive substances (TBARs)*

TBARs were used to evaluate secondary lipid oxidation products according to the modified method of Witte, Krause, & Bailet (1970). This method is based on the reaction between the thiobarbituric acid with aldehydes deriving from secondary oxidation of lipids present in meat, resulting in a colored complex that can be measured spectrophotometrically. Briefly, 8 mL of phosphate buffer aqueous solution at pH 7 were added to 2 g of meat in a 25 mL Sovirel tube and the resulting mixture was homogenized using an Ultra-Turrax T 25 BASIC (Ika-Werke, Staufen, Germany). Two mL of a 30% (v/v) TCA aqueous solution was then added to the sample mixture, homogenized, and filtered. Five mL of 0.02 M aqueous solution of TBA was added to 5 mL of the resulting sample solution in capped tubes, which were kept at 90  $^{\circ}\text{C}$  for 20 min and then maintained at 4  $^{\circ}\text{C}$  for 30 min. After centrifugation, the absorbance of the supernatant was measured at 530 nm with a UV spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan). For the quantitative determination of TBARs, a 1,1,3,3-tetramethoxypropane

standard calibration curve was used with a concentration range of  $1.89\text{-}141.50 \times 10^{-10}$  mol/mL ( $y = 0.0072 + 0.0077 x$ ;  $r^2 = 0.999$ ). The TBARS value was expressed as mg of malondialdehyde (MDA)/kg of sample. Two replicates were run per sample.

### *3. Protein carbonyl content*

Protein carbonyl groups were evaluated by derivatization with DNPH, as described by Levine et al. (1990). One g of sample was minced and homogenized in 10 mL of pyrophosphate buffer solution (pH 7.4) that contained 2 mM  $\text{Na}_4\text{O}_7\text{P}_2$ , 10 mM tris-maleate, 2 mM  $\text{MgCl}_2$ , 100 mM KCl, and 2 mM EGTA, by using Ultra Turrax at 12,000 rpm for 60 s. Two equal volumes of meat homogenate (0.1 mL) were precipitated with 1 mL of 20% TCA and centrifuged at 14,000 x g for 5 min at room temperature, and the supernatant was removed. For the determination of carbonyl content, 1 mL of 2 M HCl containing 0.2% DNPH was added, while for protein quantification 1 mL of 2 M HCl was added. Both samples were incubated for 1 h at room temperature in the dark and vortexed every 20 min. After incubation, 1 mL of 20% TCA was added to each tube and vortex for 30 s, then were centrifuged at 14,000 x g for 5 min and the supernatant removed. DNPH was removed by washing the pellet with 1 mL of ethanol:ethyl acetate (1:1, v/v) mixture, shaken and centrifuged for 5 min at 14,000 x g. This step was repeated three times. The pellet was finally solubilized in 2 mL of 6.0 M guanidine hydrochloride dissolved in 20 mM potassium phosphate monobasic (pH 2.3). The samples were kept at 4 °C overnight, and the final solution was centrifuged at 14,000 x g for 15 min to remove insoluble materials. Protein concentration was determined by measuring the absorbance at 280 nm in HCl treated samples against a standard calibration curve of BSA dissolved in 6 M guanidine HCl, with a concentration range of 0.2-1.0 mg/mL ( $y = 1.956 + 0.007x$ ;  $r^2 = 0.997$ ). Absorbance at



370 nm of the DNPH samples was measured. The carbonyl content in nmol/mg protein was calculated as described by Levine et al. (1994), using an absorption coefficient at 370 nm of 22,000/M cm for the formed hydrazones. Three replicates were run per sample.

### *3.6 Protein thiol content*

Sulphydryl groups (thiol content) were determined according to Nieto et al. (2013). The concentration of thiol contents in meat samples was quantified after derivatization with DTNB as suggested by Ellman (1959). One g of meat was homogenized in 25 mL of 5.0% SDS dissolved in 100 mM TRIS buffer (pH 8.0) using an Ultra Turrax at 8000 rpm for 30 sec. The homogenates were placed in a water bath at 80 °C for 30 min. After cooling, they were centrifuged at 14000 ×g for 10 min. The supernatant was diluted to a concentration of 1.5 mg/mL with the buffer used for homogenization (0.5 mL of the homogenate was diluted with 2.0 mL 5% SDS in 0.1 M TRIS buffer at pH 8.0). The dilution was analyzed according to Liu & Xiong (2000) by mixing 500 µL sample, 2 mL of 100 mM TRIS buffer (pH 8.0), and 500 µL of 10 mM DTNB dissolved in 100 mM TRIS buffer (pH 8.0). The absorbance at 412 nm was measured before addition of DTNB ( $ABS_{412\text{-before}}$ ) and after reaction with DTNB ( $ABS_{412\text{-after}}$ ) against a reference solution of 500 µL 5% SDS in 100 mM TRIS buffer (pH 8.0) and 2.50 mL of 100 mM TRIS buffer (pH 8.0). The mixture was allowed to react protected against light for exactly 30 min. A solution containing 2 mL 100 mM TRIS buffer (pH 8.0), 500 µL 5% SDS and 500 µL 10 mM DTNB was used as blank sample ( $ABS_{412\text{-blank}}$ ). The protein concentration was determined spectrophotometrically at 280 nm using a standard curve prepared from BSA dissolved in 5.0% SDS in 100 mM TRIS buffer (pH 8.0) with a concentration range of 0.3-3.0 mg/mL ( $y = 1.645 + 0.007x$ ;  $r^2 = 0.999$ ). The corrected absorbance was determined by:  $Abs\ change = ABS_{412\text{-after}} - ABS_{412\text{-before}} - ABS_{412\text{-blank}}$

blank. The final thiol concentration was calculated standard calibration curve prepared from L-cysteine diluted in 5.0% SDS in 100 mM TRIS buffer (pH 8.0), with a concentration range of 5-200  $\mu$ M thiols ( $y = 583.2 + 26.65x$ ;  $r^2 = 0.993$ ). The thiol content was calculated in nmol thiol/mg protein. Three replicates were run per sample.

#### **4. Statistical analysis**

The data are reported as mean values of three independent replicates (n=3) for each treatment group (total n=15). Tukey's honest significance test was performed at a 95% confidence level ( $p \leq 0.05$ ), in order to separate means of statistically difference. Pearson correlation coefficients ( $\alpha=0.05$ ) were used to examine possible relationships between oxidative parameters. Statistical analysis of the data was performed by SPSS 20.0.0 (2011, IBM-SPSS Inc., Chicago, Illinois, USA).

#### **5. Results and discussion**

##### *5.1 Lipid content*

Lipid content of beef loin and chicken breast meats (1.9-2.3%; 1.60-1.74%, respectively) was similar ( $p>0.05$ ) among treatments (data not shown). Beef loin meat had higher total fat content than chicken breast meat, which is in agreement with data reported by Faustman et al. (2010). It must be pointed out that the fat composition in meat is more important than its fat content, because the susceptibility of muscle lipids to oxidation varies upon polyunsaturation degree of fatty acids (Min et al., 2008).

##### *5.2 Total fatty acid composition*

Tables 1 and 2 show the FA composition of total lipids in beef loin and chicken breast meats (mean values expressed as g/100 g of lipids). In beef meat (Table 1), the main FA was linoleic acid (~ 23-27% of total FA; 9.8-11.2 g/100 g of lipids), followed by palmitic (~ 20-22%; 8.3-9.3 g/100 g of lipids), oleic (~ 19-22%; 7.9-9.3 g/100 g of lipids) and stearic acids (~ 15-16%, 6.3-7.0 g/100 g of lipids). Among long-chain PUFA, arachidonic acid (~ 3-5%, 1.6-2.5 g/100 g of lipids) was the most abundant, whereas docosahexaenoic (DHA) was detected at trace levels. In chicken breast meat (Table 2), the main FA was linoleic acid (~ 30% of total FA; 19.55-20.29 g/100 g of lipids), followed by oleic (~ 25-26%; 16.75-17.06 g/100 g of lipids), palmitic (~ 19-20%; 13.05-13.26 g/100 g of lipids) and stearic acids (~ 8%, 5.43-5.47 g/100 g of lipids). Among long-chain PUFA, arachidonic acid (~ 3%, 1.96-2.41 g/100 g of lipids) was the most abundant, whereas docosahexaenoic (DHA) was detected at trace levels.

In general, beef meat FA composition was partially ( $p < 0.05$ ) affected by storage conditions and exposure to light, while no significant effects ( $p > 0.05$ ) were observed in the FA composition of chicken breast meat.

**Table 1.** Fatty acid composition of raw beef meat (expressed as g/100 g of lipids) as related to storage conditions.

Fatty acids	T0		T5D		T5L		Stat. sig.
	Mean	SE	Mean	SE	Mean	SE	
C14:0	0.41	0.08	0.49	0.06	0.48	0.07	ns
C14:1	0.03	0.01	0.04	0.01	0.04	0.01	ns
C15:0	0.12	0.01	0.14	0.01	0.14	0.01	ns
C16:0	8.33	0.05	9.05	0.40	9.35	0.15	ns
C16:1t	0.06	0.00	0.05	0.00	0.07	0.01	ns
C16:1c7	0.47	0.00	0.53	0.00	0.59	0.00	ns
C16:1 c9	0.07	0.05	0.07	0.04	0.08	0.05	ns
C17:0	0.31	0.03	0.33	0.02	0.33	0.01	ns
C17:1	0.16	0.00	0.17	0.01	0.18	0.01	ns
C18:0	6.26	0.18	7.02	0.30	7.04	0.07	ns
C18:1 t	0.44	0.02	0.54	0.04	0.53	0.02	ns
C18:1 c9	7.85	0.20	8.76	0.47	9.25	0.66	ns
C18:1 c11	1.10	0.04	1.10	0.03	1.15	0.09	ns
C18:2 (n-6)	11.24	0.33	10.47	0.52	9.76	1.22	ns

C20:0	0.02	0.00		0.03	0.00		0.03	0.00		ns
C18:3 (n-6)	0.04	0.00		0.03	0.00		0.03	0.00		ns
C18:3 (n-3)	0.27	0.01		0.24	0.01		0.22	0.02		ns
C20:1	0.05	0.00		0.05	0.01		0.05	0.00		ns
CLA	0.06	0.00		0.06	0.01		0.07	0.01		ns
C20:2 (n-6)	0.09	0.00		0.09	0.00		0.09	0.01		ns
C22:0	0.08	0.01		0.07	0.00		0.06	0.01		ns
C20:3	0.60	0.02		0.58	0.03		0.46	0.07		ns
C20:4 (n-6)	2.36	0.11	a	2.53	0.13	a	1.56	0.23	b	0.014
C20:5 (n-3)	0.16	0.01	a	0.17	0.01	a	0.10	0.01	b	0.005
C22:4 (n-6)	0.27	0.01		0.28	0.02		0.19	0.03		ns
C22:5 (n-3)	0.46	0.02	a	0.48	0.03	a	0.30	0.04	b	0.010
C22:6 (n-3)	0.03	0.00	a	0.03	0.00	a	0.02	0.00	b	0.018

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T5D, samples stored at dark for five days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

**Table 2.** Fatty acid composition of raw chicken breast meat (expressed as g/100 g of lipids) as related to storage conditions.

Fatty acids	T0		T5D		T5L		Stat. sig.
	Mean	SE	Mean	SE	Mean	SE	
C14:0	0.24	0.01	0.25	0.01	0.25	0.01	ns
C14:1	0.05	0.01	0.05	0.01	0.05	0.01	ns
C15:0	0.04	0.00	0.04	0.00	0.04	0.00	ns
C16:0	13.05	0.88	13.26	0.59	13.22	0.66	ns
C16:1c7	0.17	0.00	0.16	0.01	0.17	0.01	ns
C16:1 c9	1.48	0.29	1.44	0.28	1.48	0.30	ns
C17:0	0.09	0.00	0.09	0.00	0.09	0.01	ns
C17:1	0.03	0.00	0.03	0.00	0.03	0.00	ns
C18:0	5.43	0.04	5.47	0.22	5.46	0.18	ns
C18:1 t	0.09	0.01	0.09	0.00	0.09	0.00	ns
C18:1 c7	16.96	1.10	16.75	1.05	17.06	1.17	ns
C18:1 c11	1.44	0.13	1.46	0.11	1.46	0.10	ns
C18:2 (n-6)	20.29	0.93	19.55	1.14	20.13	1.37	ns
C20:0	0.03	0.00	0.03	0.00	0.03	0.00	ns
C18:3 (n-6)	0.12	0.03	0.12	0.03	0.12	0.03	ns
C18:3 (n-3)	1.81	0.13	1.70	0.16	1.78	0.18	ns
C20:1	0.18	0.01	0.18	0.01	0.18	0.01	ns
CLA	0.05	0.01	0.04	0.01	0.05	0.01	ns
C20:2 (n-6)	0.46	0.04	0.46	0.06	0.46	0.05	ns
C22:0	0.04	0.00	0.04	0.01	0.04	0.01	ns
C20:3 (n-6)	0.42	0.01	0.39	0.01	0.41	0.01	ns
C20:3	0.08	0.03	0.08	0.04	0.08	0.05	ns
C20:4 (n-6)	2.41	0.18	1.96	0.28	2.19	0.35	ns
C20:5 (n-3)	0.14	0.01	0.11	0.01	0.13	0.02	ns
C22:4 (n-6)	0.63	0.06	0.54	0.10	0.59	0.10	ns
C22:5 (n-3)	0.66	0.03	0.53	0.08	0.60	0.08	ns
C22:6 (n-3)	0.30	0.03	0.20	0.03	0.24	0.03	ns

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T5D, samples stored at dark for five days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

**Table 3.** Total saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (expressed as % of total FA), and n-6/n-3 in raw beef meat, as related to storage conditions.

	T0		T5D		T5L		Stat. sig.			
	Mean	SE	Mean	SE	Mean	SE				
Σ SFA	37.58	0.50	39.41	1.15	41.41	1.24	ns			
Σ UFA	62.30	0.51	60.47	1.16	58.43	1.25	ns			
Σ MUFA	24.73	0.78	26.04	0.89	28.38	2.15	ns			
Σ PUFA	37.55	0.78	34.41	2.05	30.05	3.36	ns			
ΣSFA/Σ UFA	0.60	0.00	b	0.63	0.03	ab	0.73	0.03	a	0.031
n-3	2.23	0.06	a	2.15	0.11	a	1.50	0.15	b	0.007
n-6	35.33	0.73		32.23	1.94		28.53	3.22		ns
n-6/n-3	15.82	0.11	b	15.01	0.31	b	19.01	0.24	a	0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T5D, samples stored at dark for five days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

**Table 4.** Total saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (expressed as % of total FA), and n-6/n-3 in raw chicken breast meat, as related to storage conditions.

	T0		T5D		T5L		Stat. sig.
	Mean	SE	Mean	SE	Mean	SE	
Σ SFA	20.97	0.61	21.82	0.37	21.22	0.42	ns
Σ UFA	79.03	0.62	78.18	0.38	78.77	0.43	ns
Σ MUFA	69.53	0.52	69.85	0.83	69.94	1.17	ns
Σ PUFA	9.49	0.62	8.32	0.82	8.84	0.96	ns
ΣSFA/Σ UFA	0.27	0.03	0.30	0.00	0.30	0.00	ns
n-3	1.94	0.15	1.58	0.24	1.73	0.25	ns
n-6	7.53	0.47	6.73	0.58	7.1	0.70	ns
n-6/n-3	3.90	0.06	4.35	0.27	4.17	0.20	ns

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T5D, samples stored at dark for five days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

After 5 days of chill storage of beef meat, exposure to light led to a significant decrease ( $p < 0.05$ )

on n-3 FA, as well as to a significant increase ( $p < 0.05$ ) on both ΣSFA/ΣUFA and n-6/n-3 FA

ratios. Photooxidation mainly affected n-3 PUFAs, since arachidonic, eicosapentaenoic,

docosapentaenoic and docosahexaenoic acids significantly decreased; however, no storage effect was noted as no significant differences on n-3 PUFAs were detected between T0 and T5D samples.

The n-3 and n-6 FAs play an important role in human nutrition, both being precursors of eicosanoids, prostaglandins, leucotrienes, and thromboxanes that regulate the cardiovascular system and immunological processes (Grashorn, 2007). According to joint statements by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) (WHO, 2008), the recommended ratio of PUFA and SFAs in diets should be between 0.4 and 1.0, while n-6/n-3 PUFA ratio should be between 1 and 4 (Patterson et al., 2012). In the present study, the n-6/n-3 FA ratio was 3.9-4.1 for chicken breast and 15-19 in beef loin, the latter being much higher than the nutritional recommendation ratio. The former is a characteristic trait of current western diets, which are usually deficient in n-3 PUFAs (especially long chain FA) and contain excessive amounts of n-6 PUFAs (Simopoulos, 2006).

### 5.3 Lipid oxidation

The effect of light exposure on lipid oxidation of beef loin and chicken breast meat was evaluated by PV and TBARs, as shown in Tables 5 and 6.

In beef loin (Table 5), PV ranged from 8.56 (T0) to 21.04 (T5L) meq O<sub>2</sub>/kg of lipids, exhibiting a significant ( $p < 0.05$ ) increase due to both storage time and light exposure. These PV data are much higher than those reported by Cardenia et al. (2015), who exposed beef (*longissimus lumborum* (LL)) slices to light for a shorter time period (only 8 h at 8 °C). Beef samples kept at dark (TD5) displayed a 2.2 times higher PV level than fresh meat (T0), which could be due to the simultaneous action of both autoxidation and enzymatic oxidation mechanisms during storage

(Boselli et al., 2012). T5L samples showed the highest PV, as white fluorescent light promotes peroxide formation. The evident increase of PV during storage when kept under light or darkness indicates that the rate of formation of lipid hydroperoxides is probably faster than that of their decomposition. All samples stored for 5 days were close or above the threshold of 20 meq of O<sub>2</sub>/kg of lipids associated with oil rancidity (Ripoll et al., 2011). Regarding TBARs in beef slices, it varied from 1.1 to 3.02 mg malondialdehyde (MDA)/kg meat, which corresponded to T0 and T3L, respectively. During storage, TBARs significantly ( $p<0.05$ ) rose from 1.1 (T0) to 2.87 (T5D) mg MDA/kg meat, while they showed a bell-shape trend during light exposure with a significant ( $p<0.05$ ) drop to 2.45 mg MDA/kg meat in T5L. It might be possible that part of the secondary oxidation products reacted with other molecules (such as proteins or DNA), resulting in higher rate of TBARs reaction than that of their formation; in fact, the secondary products derived from lipid oxidation can interact with the amino acids of proteins, and malonylaldehyde is known to react with histidine and lysine residues of proteins to form stable adducts (Zarkovic et al., 2013). In the present study, the TBARs level of all beef samples stored for 3 and 5 days were above the threshold of 2 mg MDA/kg beef (Campo et al., 2006). Likewise PV, the TBARs data here found are higher than those reported by Cardenia et al. (2015), due to the longer light exposure and storage time here employed.

**Table 5.** Effects of light exposure and storage conditions on the average of peroxide value (PV, meq O<sub>2</sub>/kg fat) and TBARs (mg MDA/kg meat) of raw beef meat.

	T0		T3D		T5D		T3L		T5L		Stat. Sig.
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
PV	8.56	0.34	14.56	0.38	19.02	0.40	16.54	0.46	21.04	0.38	<0.001
TBARs	1.1	0.13	2.03	0.06	2.87	0.04	3.02	0.09	2.45	0.06	<0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T3D, samples stored at dark for three days; T5D, samples stored at dark for five days; T3L, samples exposed at light for three days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, d, statistically different means (Tukey's test;  $p\leq 0.05$ ).

In chicken breast meat (Table 6), primary lipid oxidation also occurred under the conditions used, but to a diverse extent with respect to beef loin. PV in chicken meat varied from 2.78 to 3.96 meq O<sub>2</sub>/kg of lipids, which corresponded to T0 and T5D, respectively. During storage, PV significantly ( $p<0.05$ ) decreased at T3D, but it showed a significant increase at T5D, which is the characteristic bell-shape behavior of primary oxidation products; after exposure to light, PV significantly ( $p<0.05$ ) decreased with increasing time of light exposure 3.2 (T3L) to 1.76 (T5L) meq O<sub>2</sub>/kg of lipids) due to hydroperoxide breakdown induced by light and the consequent conversion into secondary oxidation products. In fact, TBARs varied from 0.09 (T0) to 0.21 (T5L) mg MDA/kg meat, evidencing a significant ( $p<0.05$ ) increase due to both storage time and light exposure. It must be noted that the level of lipid oxidation of all chicken breast samples were below the threshold of 0.5-1 mg MDA/kg of white meat (Smet et al., 2008). However, data found in the present study were higher than those reported by Funaro et al. (2014) for conventional and free-range chickens, which could be attributed to diverse type of feeding, animal breed, species, muscle type and anatomical location (Nute et al., 2007; Min et al., 2008).

**Table 6.** Effects of light exposure and storage conditions on the average of peroxide value (PV, meq O<sub>2</sub>/kg fat) and TBARs (mg MDA/kg meat) of raw chicken breast meat.

	T0		T3D			T5D			T3L			T5L			Stat. Sig.	
	Mean	SE		Mean	SE	cd	Mean	SE	a	Mean	SE	b	Mean	SE		d
PV	2.78	0.02	bc	2.10	0.12	cd	3.96	0.11	a	3.20	0.07	b	1.76	0.11	d	<0.001
TBARs	0.09	0.01	c	0.13	0.01	bc	0.17	0.00	ab	0.14	0.00	b	0.21	0.02	a	<0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T3D, samples stored at dark for three days; T5D, samples stored at dark for five days; T3L, samples exposed at light for three days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, d, statistically different means (Tukey's test;  $p\leq 0.05$ ).

Moreover, the type of diet consumed by animals during the production phase has a great influence on the meat susceptibility to postmortem oxidation (Zhang et al., 2011). The oxidative



status of dietary fat can have also a significant impact on the storage stability of raw chicken meats (Racan Ricci et al., 2008; Zouari et al., 2010).

As confirmed by these results, beef has been found to be more susceptible to lipid oxidation than poultry, as PV and TBARs were about 12 times higher after 5 days of light exposure. These differences could be mainly ascribed to the larger amounts of iron and heme pigments in beef muscles (Lund et al., 2007a, b), which act as pro-oxidants and photo-sensitizers, respectively, thus greatly impacting the nutritional and sensory quality of meat.

#### *5.4 Protein oxidation*

The effect of light exposure on protein oxidation in beef loin and chicken breast meat was evaluated by means of both carbonyl and thiol contents (see Tables 7 and 8). The side chains of some particular amino acids (such as arginine, lysine and proline) are oxidized through metal-catalyzed reactions into carbonyl residues, while others (such as cysteine or methionine) are involved in cross-linking or yield sulfur containing derivatives. The secondary products derived from lipid oxidation can interact with the amino acids of proteins, and these interactions can regulate protein structure and function (Zhang et al., 2011).

In beef loin (Table 7), the carbonyl content ranged from 5.42 (T0) to 8.11 (T5L) nmol carbonyl/mg of protein and it significantly ( $p < 0.05$ ) increased due to both storage time and light exposure. The relatively high carbonyl content in this storage experiment indicates that the beef meat was already oxidized to some extent before chill storage; this could be attributed to the Italian slaughtering and processing practices, which imply a holding period of several days at 3–6 °C, aimed at improving meat tenderness and promoting the formation of aroma compounds or their precursors (Rodriguez-Estrada et al, 1997). Furthermore, light exposure can accelerate

protein oxidation. This result is in agreement with the finding of Popova et al. (2009) who reported that carbonyl content increased in beef *Longissimus dorsi* muscles stored during 6 days in darkness at 4°C. In other studies, basal level of carbonyl groups has been reported in fresh beef meat to be approximately 3 nmol/mg protein (Martinaud et al., 1997; Mercier et al., 2004). Regarding thiol content in beef loin, it varied from 95.6 to 79 nm thiol/mg of protein, which corresponded to T0 and T5L, respectively. Thiol content significantly ( $p<0.05$ ) decreased due to both storage conditions (time and light exposure). The results of the present study are in agreement with the finding of Zakrys-Walliwander et al. (2012), who reported that free thiol content decreased in beef *Longissimus dorsi* muscles packed under vacuum and stored at 4°C for 8 and 14 days. The thiol group of cysteine (RSH) is highly susceptible to oxidation in the presence of hydrogen peroxide, which is formed in cells and accumulated in meat post-mortem (Lund et al., 2011). The derivatives of interactions between lipid oxidation products and amino acid residues can cause formation of cross-linkage between proteins (Zamora et al., 2000); the formation of disulfide cross-links has been observed in fresh meat (Lund et al., 2007a; Kim et al., 2010).

**Table 7.** Effects of light exposure and storage conditions on the average of carbonyl content (nm carbonyl/mg protein), and thiol content (nm thiol/mg protein) of raw beef meat.

	T0			T3D			T5D			T3L			T5L			Stat. Sig.
	Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE		
Carbonyl	5.42	0.11	d	6.24	0.10	c	7.23	0.05	b	7.20	0.13	b	8.11	0.08	a	<0.001
Thiol	95.6	0.80	a	90.2	0.55	b	82.2	0.69	d	86.2	0.44	c	79.0	0.32	e	<0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T3D, samples stored at dark for three days; T5D, samples stored at dark for five days; T3L, samples exposed at light for three days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, d, e, statistically different means (Tukey's test;  $p\leq 0.05$ ).

As observed for lipid oxidation, a lower extent of protein oxidation was found in chicken breast meat (Table 8) with respect to beef loin. In fact, carbonyl content ranged from 3.03 (T0) to 3.87 (T5L) nm carbonyl/mg of protein and it significantly ( $p<0.05$ ) rose due to both storage conditions (time and light exposure). The data here found were lower than those reported by Wang et al. (2009) in broiler muscle pectoralis major at slaughter, which could be due to many factors including meat origin, muscle type, species, feeding, breeding, slaughtering and storage conditions (Lund et al., 2007a,b; Santé-Lhoutellier et al., 2008; Filgueras et al., 2010; Jung et al., 2010). The oxidative reactions occurring in muscle can result in the generation of carbonyls (aldehydes and ketones), protein polymers, and peptide scissions (Ooizumi and Xiong, 2004). Among them, formation of carbonyls is one of the most prominent changes in oxidized proteins, and the content of carbonyl is widely used as a marker of protein damage (Lund et al., 2008). Thiol content varied from 84.57 to 78.23 nm thiol/mg of protein, which corresponded to T0 and T5L, respectively. A significant ( $p<0.05$ ) decrease of thiol content was also due to both storage time and exposure to light. These values of thiol content are higher than those found in frozen-thawed chicken breast meat (Ali et al., 2015); the losses of thiol groups depend on the type of muscle, experimental conditions and species (Lund et al., 2011).

**Table 8.** Effects of light exposure and storage conditions on the average of carbonyl content (nm carbonyl/mg protein), and thiol content (nm thiol/mg protein) of raw chicken breast meat.

	T0		T3D		T5D		T3L		T5L		Stat. Sig.					
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE						
Carbonyl	3.03	0.05	d	3.14	0.04	cd	3.44	0.06	b	3.27	0.02	bc	3.87	0.04	a	<0.001
Thiol	84.6	0.27	a	83.0	0.12	b	80.8	0.23	c	81.8	0.38	c	78.2	0.17	d	<0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: T0, fresh raw meat; T3D, samples stored at dark for three days; T5D, samples stored at dark for five days; T3L, samples exposed at light for three days; T5L, samples exposed at light for five days; Stat. signif., statistical significance; a, b, c, d, statistically different means (Tukey's test;  $p\leq 0.05$ ).

As already stated, beef was found to be more susceptible to protein oxidation than poultry, as carbonyls were about 2 times higher after 5 days of light exposure and thiols displayed a more pronounced decreasing rate (17% vs. 7.5%). These differences could be mainly ascribed to the greater extent of lipid oxidation observed in beef loin. Mercier et al. (1998) reported that protein oxidation seems to be influenced by the level of lipid oxidation in meat. In fact, primary and secondary lipid oxidation products can act as substrates for protein oxidation, so once the oxidation of lipids starts, the oxidation of proteins will also occur but with diverse reaction kinetics (Estevez et al., 2011). Recent studies have demonstrated that proteins can be directly attacked by reactive oxygen substances (ROS) or indirectly react with secondary lipid by-products of oxidative stress (Estévez, 2011).

### *5.5 Correlations*

A correlation study (Pearson's test,  $\alpha=0.05$ ) was performed on the results obtained for lipid and protein oxidation data from the two types of meats. For better data comprehension, only significant correlations are here discussed.

Good correlations were found between lipid and protein oxidation in beef and chicken breast meat when exposed to light under chill storage. In fact, TBARs and carbonyl content were directly correlated in beef loin ( $r = 0.788$ ) and chicken breast meat ( $r = 0.928$ ), as well as between TBARs and sulphhydryl content ( $r = 0.766$  in beef loin;  $r = 0.909$  in chicken breast meat). As stated before, both primary (hydroperoxides) and secondary (aldehydes) lipid oxidation products can react with proteins, thus promoting their oxidation (Zhang et al., 2011). Similar correlation was observed in chicken breast meat during frozen storage at  $-20\text{ }^{\circ}\text{C}$  up to 13 months (Soyer et al., 2010).

## **6. Conclusions**

Photooxidation of lipids and proteins in raw chicken breast and beef meats was studied under different storage conditions. In general, both lipid and protein oxidation parameters increased during storage at 4°C under darkness conditions, with a greater impact when exposed to light, especially in beef meat. The latter, in fact, exhibited much higher lipid and protein oxidation (about 12 and 2 times, respectively) than chicken breast. This study evidences that storage and packaging conditions can greatly affect both lipid and protein oxidations of chicken breast and beef meats, thus suitable strategies (i.e. film types and/or active packaging) should be found to improve their oxidative stability and reduce the impact of light exposure.

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## **4.2 Effect of plant extracts mixture on the oxidative stability of chicken breast meat during chill and frozen storage**

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## **Abstract**

The effects of the addition of antioxidant from plant extract mixture on lipid and protein oxidation of chicken breast meat subjected to chill and frozen storage, were investigated. Broilers were fed either a diet supplemented with or without plant extract mixture (0.35 g/kg feed) for 42 days. Freshly prepared samples were stored for short-term storage at 4 °C for 0 and 7 days, then packed in plastic bags under vacuum and subjected to long-term frozen storage at -18 °C for one year. No effect of the antioxidant plant extract mixture on lipid oxidation in fresh samples stored at 4 °C was observed. After frozen storage at -18 °C for 1-year, samples obtained with dietary antioxidant supplementation had significantly ( $p < 0.05$ ) lower PV, TBARs and carbonyls contents and higher values of n-3 PUFA and sulfhydryl content than the basal diet meat. These results suggest that dietary antioxidants can minimize the oxidative stability of both lipids and proteins in chicken breast meat during long-term frozen storage.

*Keywords:* Plant extracts; Lipid oxidation; Protein oxidation; Fatty acids composition; Shelf-life



## **1. Introduction**

Poultry meat is characterized by its high concentration of polyunsaturated fatty acids (PUFA), low lipid content and low content of natural antioxidants (Nkukwana et al., 2014; Barroeta, 2007; Grashorn, 2007). Therefore, it is highly influenced by lipid oxidation, which leads to high risk of meat deterioration and shelf life reduction. There are several factors that affects meat oxidative stability and nutritional characteristics, such as type of feed, birds genotype, muscle type, feeding conditions and the slaughter techniques (Min et al., 2008; Jung et al., 2010; Sirri et al., 2011; Zhang et al., 2011).

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ), play an important role as antioxidants in food market; however, due to consumer concern about the safety and toxicity of synthetic antioxidants, recent trends are in favor of natural antioxidants, especially from plant origin (Fasseas et al., 2007; Li et al., 2010; Hillmann 2010; Karre et al., 2013). In fact, plants, such as culinary herbs, spices and vegetables, are good sources of valuable bioactive substances, including natural antioxidants (Tayel & El-Tras, 2012; Shahidi & Zhong, 2010). Different plant products are being evaluated as natural antioxidants to preserve and improve the overall quality of meat and meat products. Most of the antioxidative potential of herbs and spices is due to the redox properties of their phenolic compounds, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Hakkim et al., 2007). Some plant extracts contain phenolic compounds (the major constituents of plant materials) that have anti-inflammatory, antimicrobial and antioxidant activities, therefore potentially effective in reducing lipid oxidation and rancidity and extending shelf-life in a large variety of poultry-based foods in muscle foods (Pennington & Fisher, 2009; Pereira et al., 2009; Hwang et al., 2013; Bekhit et al.,

2013). Several advantages could be attained applying such natural plant extracts to animal feeds, such as the growth stimulation of beneficial bacteria and the minimization of pathogenic bacterial activity in the gastrointestinal tract of poultry (Langhout, 2000; Wenk, 2000). Strategies based on dietary antioxidants may also alleviate the impact of other sources of oxidative stress in broilers (i.e., heat induction) and therefore, inhibit the negative influence of this stress on production rates (Ismail et al., 2013). Other researchers shows that plant based compounds improves the quality and shelf-life of poultry meat products as they protect against the oxidative damage occurred during frozen storage of meat and, as a result, minimize the potential negative impact of such oxidative stress and discoloration in some types of meat (Camo et al., 2008; Fasseas et al., 2007; Zinoviadou et al., 2009). Further studies should be concentrated on the combination and application of different natural antioxidants to reduce meat spoilage and to extend its storage time, as these will greatly help reducing financial losses and labor costs, and at the same time ensuring safety and improving the functional properties of the meat. However, the concentration of antioxidant compounds in plant materials varies considerably depending on genetic and environmental factors and hence their dosage application in diets and meat products varies from plant to plant (Moyo et al., 2012; Nkukwana et al., 2014).

Adaptogenic herbs, like Ashwagandha, tulsi, amla, and ginseng, are being used as anti-stress factors in human and animal medicines with proven results (Ranade & Desai, 2005; Scartezzini & Speroni, 2000). Ashwagandha (*Withania somnifera*) contains many bioactive compounds, such as withanolides, somnital glucose, withanone, di-hydroxy kaempferol-3 and rutinoides (Murthy et al., 2009; Pal et al., 2012), and it has been reported to possess immunomodulatory, general tonic, hepato-protective, anti-stress, growth promoter and antioxidant properties (Ansari et al., 2008; Singh et al., 2010; Kushwaha et al. 2012; Verma et

al., 2012). Regarding broilers, several positive effects have been observed on animal performance (Akotkar et al. 2007), body weight (Khobragade, 2003), immunomodulation (Lokhande et al., 2009), antioxidant (Kaur et al., 2003), hepatoprotective (Harikrishnan et al., 2008), antibacterial (Owais et al., 2005) effects, and reduce heat stress in broilers during summer season (Vasanthakumar et al., 2015).

*Emblica officinalis* (Amla) is a useful antioxidant, anti-inflammatory, and with adaptogenic activity, immunogenic and growth-stimulating (Sapkota et al., 2005, Wadhwa et al., 2007). Among various herbal preparations screened by Vasanthakumar et al. (2012), Amla followed by ashwagandha (*Withania somnifera*) were found to increase the body weight, immune status, serum antioxidant levels and general health of commercial broiler birds.

The mango (*Mangifera indica* L) plant has been the focus of attention of many researchers for potent antioxidants. Parts of the mango, such as stem bark, leaves and pulp are known for various biomedical applications, including antioxidative and free radical scavenging (Ajila et al., 2007; Ribeiro et al., 2007; Barreto et al., 2008), anti-inflammatory (Hernandez et al., 2007), anticancer (Percival et al., 2006), antidiabetic, anti-oxidant, anti-viral, cardiogenic, hypotensive, anti-inflammatory properties (Shah et al., 2010). A commercial aqueous stem bark extract of *M. indica* L., has been reported to have anti-inflammatory, immunomodulatory and antioxidant activities (Makare et al., 2001; Garrido et al., 2005). Singh et al. (2004) and Mahattanatawee et al. (2006) reported that mango has polyphenols (such as mangiferin, quercetin, kaempferol, gallic acid, caffeic acid, catechins, tannins), as well as vitamins E and C, all bioactive compounds with significant antioxidant activity. In the field of antioxidants, a previous study showed that the extract from stem bark of *M. indica* could reduce the production of reactive oxygen species by peritoneal macrophages in mice (Garcia et al., 2002).

Various studies on *Ocimum sanctum* (tulsi) have revealed that it has growth promoting, hypo-tensive, cardiac depressant, smooth muscle cell relaxant, antiseptic properties and anti-stress activities in broilers (Alom et al., 2015). Tulsi contains several phytochemicals that provide antioxidant as well as anti-infective and immune enhancing properties (Mohan et al., 2011). Lalit et al. (2011) concluded that the aqueous extract of *Ocimum sanctum* mixed with control diet for eight weeks shows reduction in fasting blood glucose, serum lipid profile, lipid peroxidation products, and improvement in glucose tolerance when tested on diabetic rats. The aqueous extract also increased antioxidant enzymes, like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione transferases (GT). The chemical composition of Tulsi is highly complex, as it contains many nutrients and other bioactive compounds, such as biophenols (i.e. rosmarinic acid), flavonoids (apigenin, cirsimaritin, isothymusin and isothymonin) and other components (i.e. propanoic acid). Reedy et al. (2009) concluded that dietary supplementation of 0.5% tulsi in combination with selenium (0.3 ppm) combat oxidative stress in broilers, thereby increasing the antioxidative enzyme levels (SOD, GSH-Px and CAT). To our knowledge, the combined effect of the four plants extract mixture (*Ocimum sanctum*, *Embllica officinalis*, *Withania somnifera* and *Mangifera indica*) on the oxidation level of short- and long-term stored chicken meat from animals reared under heat stress condition, has not been investigated yet.

The objective of this study is to investigate the effectiveness of a dietary plant extract mixture in contrasting oxidation level of chicken breast meat, from animals reared under heat stress condition, after being subjected to chill and frozen storage.

## **2. Materials and methods**

## *2.1 Reagents and solvents*

All chemicals used were of analytical grade. Ammonium thiocyanate ( $\text{NH}_4\text{SCN}$ ,  $\geq 97.5\%$ ), barium chloride dihydrate ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), iron (II) sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), sodium dihydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), tri-sodium phosphate ( $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and double distilled water, were supplied by Carlo Erba (Milan, Italy). Chloroform, methanol, ethanol, hydrochloric acid ( $\text{HCl}$ , 37%), potassium chloride ( $\text{KCl}$ ), *n*-hexane and isopropanol, were purchased from Merck (Darmstadt, Germany). The standard mixture of fatty acid methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN, USA). 1,1,3,3-tetraethoxypropane (precursor for malondialdehyde (MDA)), trichloroacetic acid (TCA,  $\text{CCl}_3\text{COOH}$ ,  $\geq 99.0\%$ ), 2-thiobarbituric acid ( $\geq 98.0\%$ ), anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ,  $\geq 99.0\%$ ), sodium pyrophosphate ( $\text{Na}_4\text{O}_7\text{P}_2$   $\geq 95.0\%$ ), trizma maleate ( $\geq 99.5\%$ ), magnesium chloride ( $\text{MgCl}_2$ ,  $\geq 98.0\%$ ), ethylene glycol-bis(2-aminoethylether)-tetraacetic acid (EGTA  $\geq 97.0\%$ ), 2,4-dinitrophenylhydrazine (DNPH,  $\geq 97.0\%$ ), bovine album serum (BSA,  $\geq 96\%$ ), guanidine hydrochloride ( $\geq 99.0\%$ ), potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ,  $\geq 99.0\%$ ), ethyl acetate ( $\geq 99.7\%$ ), tris(hydroxymethyl)-aminomethane (TRIS,  $\geq 99.8\%$ ), sodium dodecyl sulfate (SDS,  $\geq 99.0\%$ ), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB,  $\geq 98.0\%$ ) and L-cysteine ( $\geq 98.5\%$ ), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Filters papers (90 and 185 mm diameter) were used (Whatman, Maidstone, England).

## *2.2 Sampling, packaging and experimental design*

The experiment was set up according to a randomized complete block design, with 2 experimental groups and 12 replications per treatment. All the chicks used in the study were obtained by the same breeder flock. A total of 1,560 1-day-old male Ross 308, obtained from the

same hatching session, were used. Since quality issues are more susceptible to occur in higher stress conditions, the chicks were housed at a high stocking density of 12 chicks/m<sup>2</sup> for a total of 72 birds per pen. The feeding program included 4 feeding phases: starter (0–11 d), grower I (12–21 d), grower II (22–35 d) and finisher (36–42 d). A common basal diet (BD) for each feeding phase was prepared; ingredients and chemical composition are detailed in Table 1. Each BD was split into two treatment (T) groups, which were prepared as follows: TC: control, basal diet; TT: basal diet supplemented with plant extract mixture 0.35 g/kg; this mixture consisted of four plants extracts (*Ocimum sanctum*, *Embllica officinalis*, *Withania somnifera* and *Mangifera indica*) addressed to help chicken to adapt to stress conditions.

**Table 1.** Basal diet (BD) composition of the two dietary groups.

<b>Ingredients</b>	<b>Starter 0-11 d</b>	<b>Grower I 12-21 d</b>	<b>Grower II 22-35 d</b>	<b>Finisher 36-42 d</b>
Corn 7.3% CP	33.73	36.59	18.55	14.48
Wheat 11.25% CP	15.00	15.00	20.00	29.99
Sorghum 10.0% CP	3.00	5.00	10.00	10.00
White corn 7.3% CP	0.00	0.00	10.00	7.51
Soybean meal HY-PRO Italian 47.5% CP	19.84	17.08	12.16	8.63
Soybean meal HY-PRO Indian 46.5% CP	5.00	5.00	5.00	5.00
Sunflower meal HY-PRO 36.0% CP - 17.5% CP	2.00	2.00	2.00	2.00
Full fat soybean 35.0% CP	10.00	10.00	15.00	15.00
Corn gluten meal 57.5% CP	4.00	2.00	0.00	0.00
Soybean oil	3.07	3.59	4.35	4.77
Limestone 39.5% Ca	0.59	0.55	0.49	0.59
Dicalcium phosphate 17.0% P 25.0% Ca	1.53	1.07	0.68	0.44
Salt 38.0% Na - 58.5% Cl	0.29	0.26	0.27	0.27
Sodium bicarbonate 27.0% Na	0.10	0.10	0.10	0.10
Coline CL 75%	0.10	0.10	0.10	0.10
Aliment MHA	0.00	0.16	0.20	0.21
Lysine liquid 50% LYS	0.43	0.37	0.24	0.23
Lysine HCL 78% LYS	0.10	0.10	0.10	0.10
DL Methionine 99%	0.33	0.17	0.13	0.10
Threonine 98%	0.16	0.14	0.12	0.11
Vitamins & Trace minerals 0.4%	0.55	0.55	0.34	0.20
NSP enzyme	0.08	0.08	0.08	0.08
Phytase enzyme 0.1%	0.10	0.10	0.10	0.10

At about 42 d of age, 2 birds per pen close to the mean body weight of the group were selected, labeled and slaughtered separately from the remaining birds. After refrigeration, the carcasses were collected and delivered to the lab for meat quality analysis. Breasts were separated from the carcasses, and skin, bones, and connective tissue were removed. Samples obtained with the two dietary supplementations, were packed in a polyethylene tray, which was wrapped with a transparent shrink film and stored for short-term storage under retail conditions at 4°C for different time periods (0 and 7 days). Thereafter, the breast samples were minced twice to ensure homogenization, packed in plastic bags under vacuum, covered with aluminum foil, and then frozen for one year at -20 °C to assess the effect of antioxidant supplementation on poultry meat subjected to long-term, stressing storage conditions.

### 2.3 Proximate analysis

Proximate analysis for moisture (Table 2), crude protein, ash, ether extract and mineral composition was performed on all experimental diets, according to the methods of the Association of Official Analytical Chemists (AOAC, 2000).

**Table 2.** Chemical composition of the two dietary groups.

Chemical composition	Starter 0-11 d	Grower I 12-21 d	Grower II 22-35 d	Finisher 36-42 d
Metabolizable energy (kcal/kg)	3,070	3,140	3,240	3,280
Dry matter, %	88.65	88.45	88.43	88.47
Protein, %	23.38	21.18	19.80	18.74
Lipids, %	7.00	7.59	9.06	9.34
Fiber, %	2.85	2.82	2.88	2.86
Ash, %	5.60	5.00	4.55	4.25
Lysine, %	1.42	1.30	1.19	1.10
Methionine + Cysteine, %	1.05	0.96	0.90	0.85
Total CA, %	0.85	0.71	0.58	0.52
Total P, %	0.65	0.55	0.48	0.43
CA/ P, %	1.32	1.28	1.20	1.22
Dietary electrolytes balance (mEq/kg DM)	238	225	220	204

The techniques described by Van Soest, Robertson, and Lewis (1991) were used to determine neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations.

#### *2.4 Lipid extraction*

Lipids were extracted according to Boselli et al. (2005), a modified version of the method suggested by Folch, Lees, and Sloane-Stanley (1957). The frozen samples were minced, a subsample of 15 g was taken, and homogenized with 200 mL of a chloroform:methanol solution (1:1, v/v) in a glass bottle with screw-cap. The bottle was kept in an oven at 60 °C for 20 min before adding 100 mL chloroform. After 3 min of homogenization, the mixture was filtered through filter paper; the filtrate was mixed thoroughly with 100 mL of a 1 M KCl solution and left overnight at 4 °C to allow phase separation. The lipid-containing phase (lower) was collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Two lipid extractions were performed per each sample.

#### *2.5 Fatty acid composition*

About 20 mg of lipid extract were methylated with 200 µL of diazomethane (Fieser & Fieser, 1967); 1.11 mg of tridecanoic acid methyl ester was added (as internal standard), and the mixture was transmethylated with 40 µL of 2 N KOH in methanol (European Commission, 2002), vortexed for 1 min, kept for 5 min, and centrifuged at 1620 x g for 5 min. Supernatant was transferred to a vial before being injected into a gas chromatograph coupled to a flame ionization detector (GC-FID). The GC-FID instrument was a GC8000 series (Fisons Instruments, Milan, Italy) coupled to a computerized system for data acquisition (Chromcard Data System, ver. 2.3.1, Fisons Instruments). A Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropyl-



polysiloxane) fused-silica column (105 m × 0.25 mm × 0.2 μm film thickness) (Bellefonte, USA), was used. Oven temperature was programmed from 100 °C to 180 °C at a rate of 3 °C/min, kept at 180 °C for 10 min, and then taken to 240 °C at a rate of 3 °C/min; the final temperature was kept for 30 min. The injector and detector temperatures were both set at 250 °C. Helium was used as carrier gas at a constant pressure of 260 kPa. The split ratio was 1:50. Peak identification was carried out by comparing the peak retention times with those of the GLC 463 FAME standard mixture. The GC response factor of each fatty acid was calculated by using the GLC 463 FAME standard mixture and the internal standard (13:0). The limit of detection (LOD) of FAMES was 0.002 mg, whereas the limit of quantification (LOQ) was 0.006 mg. LOD and LOQ were calculated as signal-to-noise ratios equal to 3:1 and 10:1, respectively. Three replicates were analyzed per sample.

### *2.6 Peroxide Value (PV)*

Peroxide value was determined using a modified version of the method of Shantha and Decker (1994). In this method, peroxides oxidize ferrous ions to ferric ions, which react with ammonium thiocyanate to give rise to a colored complex that can be measured spectrophotometrically. Briefly, 20 mg of extracted lipids were mixed with 9.8 mL of chloroform:methanol (2:1, v/v) and 50 μL of thiocyanate/Fe<sup>2+</sup> solution, and then vortexed. After 5 min, the absorbance was measured at 500 nm using a double beam UV-VIS spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan). PV was calculated using a Fe (III) standard calibration curve with a concentration range of 0.599-47.95 μg/mL ( $y = 0.028 + 0.019x$ ;  $r^2 = 0.996$ ). PV was expressed as meq of O<sub>2</sub> per kg of fat. Two replicates were run per sample.

### *2.7 Thiobarbituric acid reactive substances (TBARs)*

TBARs were used to evaluate secondary lipid oxidation products according to the modified method of Witte, Krause, & Bailet (1970). This method is based on the reaction between the thiobarbituric acid with aldehydes deriving from secondary oxidation of lipids present in meat, resulting in a colored complex that can be measured spectrophotometrically. Briefly, 8 mL of phosphate buffer aqueous solution at pH 7 were added to 2 g of meat in a 25 mL Sovirel tube and the resulting mixture was homogenized using an Ultra-Turrax T 25 BASIC (Ika-Werke, Staufen, Germany). Two mL of a 30% (v/v) TCA aqueous solution was then added to the sample mixture, homogenized, and filtered. Five mL of 0.02 M aqueous solution of TBA was added to 5 mL of the resulting sample solution in capped tubes, which were kept at 90 °C for 20 min and then maintained at 4 °C for 30 min. After centrifugation, the absorbance of the supernatant was measured at 530 nm with a UV spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan). For the quantitative determination of TBARs, a 1,1,3,3-tetramethoxypropane standard calibration curve was used with a concentration range of  $1.89-141.50 \times 10^{-10}$  mol/mL ( $y = 0.0072 + 0.0077 x$ ;  $r^2 = 0.999$ ). The TBARs value was expressed as mg of malondialdehyde (MDA)/kg of sample. Two replicates were run per sample.

### *2.8 Protein carbonyl content*

Protein carbonyl groups were evaluated by derivatization with DNPH, as described by Levine et al. (1990). One g of sample was minced and homogenized in 10 mL of pyrophosphate buffer solution (pH 7.4) that contained 2 mM  $\text{Na}_4\text{O}_7\text{P}_2$ , 10 mM tris-maleate, 2 mM  $\text{MgCl}_2$ , 100 mM KCl, and 2 mM EGTA, by using Ultra Turrax at 12,000 rpm for 60 s. Two equal volumes of meat homogenate (0.1 mL) were precipitated with 1 mL of 20% TCA and centrifuged at 14,000

x g for 5 min at room temperature, and the supernatant was removed. For the determination of carbonyl content, 1 mL of 2 M HCl containing 0.2% DNPH was added, while for protein quantification 1 mL of 2 M HCl was added. Both samples were incubated for 1 h at room temperature in the dark and vortexed every 20 min. After incubation, 1 mL of 20% TCA was added to each tube and vortex for 30 s, then were centrifuged at 14,000 x g for 5 min and the supernatant removed. DNPH was removed by washing the pellet with 1 mL of ethanol:ethyl acetate (1:1, v/v) mixture, shaken and centrifuged for 5 min at 14,000 x g. This step was repeated three times. The pellet was finally solubilized in 2 mL of 6.0 M guanidine hydrochloride dissolved in 20 mM potassium phosphate monobasic (pH 2.3). The samples were kept at 4 °C overnight, and the final solution was centrifuged at 14,000 x g for 15 min to remove insoluble materials. Protein concentration was determined by measuring the absorbance at 280 nm in HCl treated samples against a standard calibration curve of BSA dissolved in 6 M guanidine HCl, with a concentration range of 0.2-1.0 mg/mL ( $y = 1.956 + 0.007x$ ;  $r^2 = 0.997$ ). Absorbance at 370 nm of the DNPH samples was measured. The carbonyl content in nmol/mg protein was calculated as described by Levine et al. (1994), using an absorption coefficient at 370 nm of 22,000/M cm for the formed hydrazones. Three replicates were run per sample.

### *2.9 Protein thiol content*

Sulfhydryl groups (thiol content) were determined according to the method described by Nieto et al. (2013). The concentration of thiol groups in meat samples was quantified after derivatization with DTNB as suggested by Ellman (1959). One g of meat was homogenized in 25 mL of 5.0% SDS dissolved in 100 mM TRIS buffer (pH 8.0) using an Ultra Turrax at 8000 rpm for 30 sec. The homogenates were placed in a water bath at 80 °C for 30 min. After cooling, they

were centrifuged at 14000 ×g for 10 min. The supernatant was diluted to a concentration of 1.5 mg/mL with the buffer used for homogenization (0.5 mL of the homogenate was diluted with 2.0 mL 5% SDS in 0.1 M TRIS buffer at pH 8.0). The dilution was analyzed according to Liu & Xiong (2000) by mixing 500 µL sample, 2mL of 100 mM TRIS buffer (pH 8.0), and 500 µL of 10 mM DTNB dissolved in 100 mM TRIS buffer (pH 8.0). The absorbance at 412 nm was measured before addition of DTNB ( $ABS_{412\text{-before}}$ ) and after reaction with DTNB ( $ABS_{412\text{-after}}$ ) against a reference solution of 500 µL 5% SDS in 100 mM TRIS buffer (pH 8.0) and 2.50 mL of 100 mM TRIS buffer (pH 8.0). The mixture was allowed to react protected against light for exactly 30 min. A solution containing 2mL 100 mM TRIS buffer (pH 8.0), 500 µL 5% SDS and 500 µL 10 mM DTNB was used as blank sample ( $ABS_{412\text{-blank}}$ ). The protein concentration was determined spectrophotometrically at 280 nm using a standard curve prepared from BSA dissolved in 5.0% SDS in 100 mM TRIS buffer (pH 8.0) with a concentration range of 0.3-3.0 mg/mL ( $y = 1.645 + 0.007x$ ;  $r^2 = 0.999$ ). The corrected absorbance was determined by:  $Abs\ change = ABS_{412\text{-after}} - ABS_{412\text{-before}} - ABS_{412\text{-blank}}$ . The final thiol concentration was calculated standard calibration curve prepared from L-cysteine diluted in 5.0% SDS in 100 mM TRIS buffer (pH 8.0), with a concentration range of 5-200 µM thiols ( $y = 583.2 + 26.65x$ ;  $r^2 = 0.993$ ). The thiol content was calculated in nmol thiol/mg protein. Three replicates were run per sample.

#### **4. Statistical analysis**

The data are reported as mean values of three independent replicates (n=3) for each treatment group (total n=15). Tukey's honest significance test was performed at a 95% confidence level ( $p \leq 0.05$ ), in order to separate means of statistically difference. Pearson correlation coefficients ( $\alpha=0.05$ ) were used to examine possible relationships between oxidative

parameters. Statistical analysis of the data was performed by SPSS 20.0.0 (2011, IBM-SPSS Inc., Chicago, Illinois, USA).

## **5. Results and discussion**

### *5.1 Lipid content*

Lipid content of chicken breast meat (1.63-1.91%) was similar ( $p>0.05$ ) among treatments (data not shown). These data are in agreement with those reported by Funaro et al. (2014). The fat composition in meat is more important than its fat content, because its susceptibility to oxidation varies according to the unsaturation degree of fatty acids (Min et al., 2008).

### *5.2 Fatty acid profile of broiler breast meat*

Table 3 shows the FA composition of total lipids in chicken breast meats (mean values expressed as g/100 g of lipids). The main FAs was linoleic acid (~ 30-32% of total FA; 21-27 g/100 g of lipids), followed by oleic (~ 24-25% of total FA; 18-21 g/100 g of lipids), palmitic (~ 22-25% of total FA; 16-17 g/100 g of lipids) and stearic acids (~ 9-10% of total FA; 7-8 g/100 g of lipids). Linoleic acid (LA; C18:2n-6) is one of the essential fatty acids and the primary precursor of all n-6 PUFAs (Russo, 2009). It is converted to arachidonic acid (AA; C20:4n-6) in animal tissues (Smith, 2008).

In general, the FA composition of chicken breast meat was affected by both dietary treatment and storage conditions. In particular, at time zero, LTT0 had a significantly ( $p<0.05$ ) higher content of C20:4 than LCT0. After frozen storage at  $-18\text{ }^{\circ}\text{C}$  for 1 year, the fatty acid composition of both types of samples were affected differently; in fact, C18:1, C18:2, C18:3 ( $\alpha$  and  $\gamma$ ) and C20:1 acids, significantly ( $p<0.05$ ) decreased in LCT7 with respect to LCT0, while

antioxidant dietary supplementation was not able to contrast the significant ( $p<0.05$ ) reduction of C22:1, C20:5n-3 and C22:5 n-3 in LTT7 with respect to LTT0. Moreover, LCT7 showed a significantly ( $p<0.05$ ) higher CLA content compared to LTT7.

**Table 3.** Effect of antioxidant dietary supplementation and long-term storage on fatty acid composition of chicken breast meat (expressed as g/100 g of lipids).

Fatty acids	LTC0			LTT0			LTC7			LTT7			Stat. Signif.
	Mean	SE		Mean	SE		Mean	SE		Mean	SE		
C10:0	0.01	0.00	ab	0.01	0.00	a	0.01	0.00	b	0.01	0.00	b	0.002
C12:0	0.02	0.00		0.02	0.00		0.03	0.00		0.02	0.00		0.746
C14:0	0.38	0.01		0.36	0.01		0.33	0.02		0.35	0.02		0.194
C14:1	0.06	0.00	ab	0.06	0.01	a	0.03	0.01	b	0.06	0.01	a	0.012
C15:0	0.07	0.00		0.07	0.00		0.07	0.01		0.06	0.00		0.144
C16:0	17.04	0.45		17.32	0.44		16.48	1.07		15.74	0.75		0.444
C16:1T	0.01	0.00		0.01	0.00		0.01	0.00		0.01	0.00		0.055
C16:1 c7	1.70	0.14	ab	2.15	0.31	a	1.01	0.15	b	1.83	0.23	ab	0.009
C17:0	0.12	0.00		0.11	0.01		0.11	0.01		0.11	0.01		0.325
C17:1	0.01	0.00	a	0.01	0.00	bc	0.01	0.00	ab	0.01	0.00	c	0.001
C18:0	7.54	0.22		7.72	0.27		6.87	0.13		6.90	0.26		0.026
C18:1t	0.11	0.00	bc	0.11	0.01	c	0.14	0.01	ab	0.16	0.01	a	0.001
C18:1 (n-9)	20.93	0.70		21.22	0.93		17.63	1.07		19.54	1.21		0.066
C18:1 (n-7)	1.53	0.03	ab	1.76	0.10	a	1.35	0.06	b	1.51	0.11	ab	0.013
C18:2 (n-6)	27.30	1.86	a	25.75	1.14	ab	21.84	0.62	b	23.11	1.24	ab	0.026
C18:3 (n-6)	0.22	0.01	a	0.23	0.02	a	0.18	0.01	b	0.22	0.01	ab	0.008
C18:3 (n-3)	1.43	0.11	a	1.43	0.09	a	1.05	0.05	b	1.39	0.09	ab	0.016
C20:1 (n-9)	0.29	0.01	a	0.29	0.02	a	0.21	0.01	b	0.26	0.02	ab	0.007
CLA	0.03	0.00	a	0.02	0.00	ab	0.03	0.00	a	0.01	0.00	b	0.002
C20:2 (n-6)	0.95	0.08		0.89	0.09		0.87	0.08		0.73	0.03		0.253
C20:3 (n-6)	0.66	0.05		0.78	0.06		0.62	0.05		0.67	0.03		0.161
C20:3 (n-3)	0.08	0.01		0.08	0.01		0.08	0.01		0.07	0.00		0.445
C22:1	3.11	0.20	ab	3.75	0.35	a	2.51	0.09	b	2.79	0.23	b	0.007
C20:5 (n-3)	0.06	0.00	ab	0.10	0.01	a	0.05	0.00	b	0.08	0.01	b	0.001
C22:4 (n-6)	0.93	0.05	b	1.14	0.11	a	0.79	0.07	ab	0.92	0.08	ab	0.036
C22:5 (n-3)	0.34	0.02	ab	0.44	0.04	a	0.26	0.01	b	0.33	0.03	b	0.001
C22:6 (n-3)	0.10	0.01	ab	0.15	0.02	a	0.09	0.01	b	0.10	0.01	ab	0.032

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: LTC0, control group (basal diet) fresh samples subjected to long-term frozen storage; LTC7, control group (basal diet) 7 days chill storage subjected to long-term frozen storage; LTT0, treatment group (basal diet supplemented with plant extracts mixture) fresh samples subjected to long-term frozen storage; LTT7, treatment group (basal diet supplemented with plant extracts mixture) 7 days chill storage subjected to long-term frozen storage; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p\leq 0.05$ ).

Table 4 shows the total saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) and the n-6/n-3 ratio in chicken breast meat, as related to antioxidant dietary

supplementation and long-term storage. Storage time significantly ( $p < 0.05$ ) reduced the contents of MUFA and PUFA (n-3 and n-6) in the basal diet sample (LCT7 vs. LCT0), giving a higher n-6/n-3 ratio. The antioxidant dietary treatment led to a significantly ( $p < 0.05$ ) higher preservation of n-3 PUFA during storage, with a reduced n-6/n-3 ratio. The n-3 and n-6 FAs play an important role in human nutrition, both being precursors of eicosanoids, prostaglandins, leucotriens, and thromboxanes that regulate the cardiovascular system and immunological processes (Grashorn, 2007).

**Table 4.** Total saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (expressed as g/100 g of lipids), and n-6/n-3 in chicken breast meat, as related to antioxidant dietary supplementation and long-term storage.

	LTC0			LTT0			LTC7			LTT7			Stat. sig.
	Mean	SE		Mean	SE		Mean	SE		Mean	SE		
Σ SFA	25.19	0.55		25.61	0.42		23.89	1.18		23.19	0.92		0.165
Σ UFA	60.13	2.45	a	60.33	1.87	a	48.75	1.31	b	53.83	2.62	ab	0.002
Σ MUFA	28.21	0.85	a	29.46	1.15	a	22.88	1.39	b	26.24	1.51	ab	0.006
Σ PUFA	31.91	1.81	a	30.87	1.34	ab	25.87	0.50	b	27.59	1.40	ab	0.014
ΣSFA/ΣUFA	0.42	0.00	b	0.43	0.00	ab	0.49	0.00	a	0.43	0.00	ab	0.029
n-6	29.92	1.73	a	28.78	1.25	ab	24.44	0.46	b	25.64	1.32	ab	0.018
n-3	1.99	0.10	a	2.09	0.11	a	1.43	0.05	b	1.95	0.10	a	0.001
n-6/ n-3	15.07	0.00	b	13.84	0.00	bc	17.19	0.00	a	13.18	0.00	c	0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: LTC0, control group (basal diet) fresh samples subjected to long-term frozen storage; LTC7, control group (basal diet) 7 days chill storage subjected to long-term frozen storage; LTT0, treatment group (basal diet supplemented with plant extracts mixture) fresh samples subjected to long-term frozen storage; LTT7, treatment group (basal diet supplemented with plant extracts mixture) 7 days chill storage subjected to long-term frozen storage; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

According to joint statements by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) (WHO, 2008), the recommended ratio of PUFA and SFAs in diets should be between 0.4 and 1.0, while n-6/n-3 PUFA ratio should be between 1 and 4 (Patterson et al., 2012). In the present study, the n-6/n-3 FA ratios ranged from 13.18-17.19, thus being much higher than the nutritional recommended ratio. Such ratio value is a characteristic

trait of current western diets, which are usually deficient in n-3 PUFAs (especially long chain FA) and contain excessive amounts of n-6 PUFAs (Simopoulos, 2006); such ratio can be improved by using suitable dietary strategies (Palmquist, 2009), that lead to an increase of long chain n-3 PUFA, such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) (Givens et al., 2011).

### *5.3 Lipid oxidation*

#### *5.3.1 Peroxide Value*

The effect of dietary supplementation of plant extracts on lipid oxidation in chicken breast meat subjected to chill and frozen storage, was evaluated by PV and TBARs (Tables 5 and 6). In chilled meat, PV values ranged from 1.00 and 1.40 meq O<sub>2</sub>/kg lipid, which corresponded to STC0 and STC7, respectively. No significant effect of dietary antioxidant on PV values was observed, being very low throughout the whole chilled storage period.

After long-term frozen storage, PV level varied from 1.32 to 1.96 mg O<sub>2</sub>/kg of lipids, which corresponded to the treated fresh raw meat (LTT0) and the basal group previously subjected to the short-term fridge storage (LTC7), respectively. PV significantly increased ( $p < 0.05$ ) in breast meat during frozen storage for 12 months for all dietary treatments, even though PV values kept still quite low. However, PV level in control samples was significantly higher than those of treatment samples, which confirms the antioxidant effect of the dietary plant mixture. In any case, lipid oxidation occurred during freezing storage at -18 °C, but at a lower rate and favoring peroxide formation rather than their conversion into secondary oxidation products (see below). Soyer et al. (2010) reported an increase in PV in chicken breast meat during frozen storage at -18 °C for 6 months. The extent of quality losses in frozen meat is dependent upon many factors,



including the rate of freezing, storage temperature, and temperature fluctuations (Muela et al., 2010).

**Table 5.** Effect of antioxidant dietary supplementation and short-term storage on the peroxide value (PV, meq O<sub>2</sub>/kg fat) and TBARs (mg MDA/kg meat) of chicken breast meat.

Formulation	STC0		STC7		STT0		STT7		Stat. Signif.
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
PV	1.00	0.03	1.40	0.10	1.30	0.10	1.32	0.17	0.078
TBARs	0.23	0.04	0.23	0.02	0.21	0.07	0.29	0.03	0.594

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: STC0, control group (basal diet) fresh samples; STC7, control group (basal diet) 7 days chill storage; STT0, treatment group (basal diet supplemented with plant extracts mixture) fresh samples; STT7, treatment group (basal diet supplemented with plant extracts mixture) 7 days chill storage samples; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

### 5.3.2 TBARs

In short-term chill storage, there was no significant effect of dietary antioxidant on TBARs values, which were very low throughout the whole storage period. TBARs values ranged from 0.21 and 0.29 mg MDA/Kg meat, which corresponded to STT0 and STT7, respectively. Data found in the present study were higher than those reported by Funaro et al. (2014) for conventional and free-range chickens, which could be attributed to diverse type of feeding, animal breed, species, muscle type and anatomical location (Nute et al., 2007; Min et al., 2008). Moreover, the type of diet consumed by animals during the production phase has a great influence on the meat susceptibility to postmortem oxidation (Zhang et al., 2011).

In contrast, long-term frozen storage TBARs values ranged from 0.59 (LTT0) and 0.75 (LTC7) mg MDA/Kg meat on day-0 and day-7 chilled stored meat subjected to further frozen storage for 12 months at -18 °C. TBARs in control samples were significantly ( $p < 0.05$ ) higher than those of treatment samples, which evidences an antioxidant effect of the dietary plant mixture. TBARs values increased significantly ( $p < 0.05$ ) during storage, but all remained below the threshold of

0.5-1 mg of MDA/Kg of meat (Smet et al., 2008). TBARs levels above 0.5 are considered critical, since they are related to the development of rancid odor and taste that can be detected by consumers (Wood et al., 2008). Data found in the present study were in agreement with those reported by Smet et al. (2008) and Soyer et al. (2010).

**Table 6.** Effect of antioxidant dietary supplementation and long-term storage on the peroxide value (PV, meq O<sub>2</sub>/kg fat) and TBARs (mg MDA/kg meat) of chicken breast meat.

Formulation	LTC0			LTC7			LTT0			LTT7			Stat. Signif.
	Mean	SE		Mean	SE		Mean	SE		Mean	SE		
PV	1.51	0.09	bc	1.96	0.08	a	1.32	0.03	c	1.70	0.05	b	0.001
TBARs	0.64	0.03	bc	0.75	0.02	a	0.59	0.02	c	0.68	0.02	ab	0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: LTC0, control group (basal diet) fresh samples subjected to long-term frozen storage; LTC7, control group (basal diet) 7 days chill storage subjected to long-term frozen storage; LTT0, treatment group (basal diet supplemented with plant extracts mixture) fresh samples subjected to long-term frozen storage; LTT7, treatment group (basal diet supplemented with plant extracts mixture) 7 days chill storage subjected to long-term frozen storage; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

Although no significant effects of the dietary supplementation of the plant extract mixture were observed during chilled storage, PV and TBARs significantly ( $p < 0.05$ ) decreased in treated meat samples during long-term frozen storage, leading to a lower extent of primary and secondary lipid oxidation. As observed by the value range of the two lipid oxidation parameters in all samples, vacuum packaging also helped controlling lipid oxidation and extending the meat shelf-life during long-term frozen storage (Veberg et al., 2006).

#### 5.4 Protein oxidation

Protein oxidation occurs through a chain reaction of free radicals like lipid oxidation of in animal muscle (Lund et al., 2011). Recent studies have demonstrated that proteins can also be vulnerable to oxidative reactions by direct attack of reactive oxygen substances (ROS) or by

indirect reactions with secondary lipid by-products of oxidative stress (Stadtman, 2004; Estévez, 2011). In fact, when proteins are targeted by ROS, this interaction leads to the generation of carbonyl compounds and the loss of sulfhydryl groups from proteins (Stadtman, 1990; Xiong, 2000; Lund et al., 2011). ROS can also attack the side chains of basic amino acids (histidine, arginine, and lysine) and can convert them into carbonyl derivatives.

#### *5.4.1 Carbonyl content*

Carbonylation is an irreversible and non-enzymatic modification of proteins that involves the formation of carbonyl groups induced by oxidative stress and other mechanisms (Estévez, 2011).

The effect of dietary supplementation of plant extracts on protein oxidation in chicken breast meat subjected to long-term frozen storage was evaluated by carbonyl and thiol contents (Table 7). In the present study, protein oxidation showed a similar behavior to that observed for lipid oxidation. After long-term frozen storage, carbonyl content ranged from 3.35 to 4.29 nmol carbonyl/mg of protein, which corresponded to LTT0 and LTC7, respectively. The values of protein carbonyl compounds here observed were lower than those previously reported in broiler pectoralis major muscle at slaughter (Wang et al., 2009). In general, the amount of protein carbonyls in breast meat increased significantly ( $p < 0.05$ ) during frozen storage for 12 months for all dietary treatments. The carbonyl level in control samples was higher than those in treated samples, which confirms that dietary antioxidant extract was also able to delay protein oxidation.

The effect of antioxidants and storage time on protein carbonyl formation was overall similar to that of TBARs, suggesting a possible relationship and interaction between lipid oxidation and protein carbonyl formation. Malondialdehyde, a secondary dicarbonyl product of

lipid oxidation, can interact with amine groups in proteins, generating protein-bound carbonyls (Estevez, 2011). Moreover, lipid and protein oxidations and their consequent interactions are accelerated by any process causing disruption of muscle cell membranes, such as size reducing processes (grinding) and freezing, as it results in exposure of lipids and proteins to oxygen and ROS.

**Table 7.** Effect of antioxidant dietary supplementation and long-term storage on Carbonyl content (nmol carbonyl/mg protein), and thiol content (nmol thiol/mg protein) of chicken breast meat.

Formulation	LTC0			LTC7			LTT0			LTT7			Stat. Signif.
	Mean	SE		Mean	SE		Mean	SE		Mean	SE		
Carbonyl	3.64	0.05	b	4.29	0.06	a	3.35	0.04	c	3.88	0.11	b	0.001
Thiol	82.23	0.37	b	72.17	0.42	d	84.89	0.33	a	78.27	0.36	c	0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: LTC0, control group (basal diet) fresh samples subjected to long-term frozen storage; LTC7, control group (basal diet) 7 days chill storage subjected to long-term frozen storage; LTT0, treatment group (basal diet supplemented with plant extracts mixture) fresh samples subjected to long-term frozen storage; LTT7, treatment group (basal diet supplemented with plant extracts mixture) 7 days chill storage subjected to long-term frozen storage; Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

#### 5.4.2 Sulfhydryl content

Protein oxidation is also associated with a decrease in sulfhydryl groups, which are converted into disulphides (Lund et al., 2011). Sulfhydryls from cysteine residues are highly susceptible to oxidation by most forms of ROS (Lund et al., 2011). However, exposure of proteins to ROS attack results in multiple chemical changes besides loss of thiol groups, including tryptophan fluorescence and formation of Schiff bases and disulfide bonds (Lund et al. 2011).

As shown in Table 7, thiol content varied from 84.89 to 72.17 nmol thiol/mg of protein, which corresponded to the control fresh raw meat LTT0 and LTC7 samples, respectively. Frozen

storage had significant ( $p < 0.05$ ) effects on the sulfhydryl content of chicken breast meat. Furthermore, samples from broilers fed the dietary plant mixture (LTT0) already had since the beginning a higher ( $p < 0.05$ ) sulfhydryl content compared with LTC0 samples, thus confirming the antioxidant effect of the supplemented diet. A decrease in sulfhydryl content in chicken meat during frozen storage, has also been reported by Soyer et al. (2010).

As noticed for lipid oxidation, both carbonyl and thiol contents were significantly ( $p < 0.05$ ) impacted by the dietary supplementation of the plant extract mixture, leading to a lower extent of protein oxidation in treated samples. Vacuum packaging during storage controlled protein oxidation of chicken breast meat, due to the decrease of lipid oxidation. The secondary products derived from lipid oxidation can interact with the amino acids of proteins, and these interactions can regulate protein structure and function (Zhang et al., 2011). The derivatives of interactions between lipid oxidation products and amino acid residues can cause formation of cross-linkage between proteins (Zamora et al., 2000).

### *5.5 Correlations*

Lipids and protein oxidation data from chicken breast meat were further analyzed by using a correlation study (Pearson's test,  $\alpha=0.05$ ). Only significant correlations are addressed for better understanding.

Lipid and protein oxidations in chicken breast meat showed a good correlation when packed in plastic bags under vacuum and subjected to long-term frozen storage at  $-18\text{ }^{\circ}\text{C}$  for one year. In fact, TBARs was directly correlated with the carbonyl ( $r = 0.601$ ) and sulfhydryl contents ( $r = 0.775$ ); PV was also directly correlated with both protein oxidation parameters ( $r = 0.772$  and  $0.809$  for carbonyl and sulfhydryl contents, respectively). The correlation analysis

clearly evidences that lipid oxidation promoted the oxidative damage of proteins through the pro-oxidant activity of primary (hydroperoxides) and secondary (aldehydes) lipid oxidation products (Li and King, 1999; Zhang et al., 2011). In addition, Soyer et al. (2010) observed similar results in chicken breast meat during frozen storage at -20 °C up to 13 months.

## **6. Conclusions**

This study shows that the dietary supplementation with antioxidants from plant extract mixture (*Ocimum sanctum*, *Emblica officinalis*, *Withania somnifera* and *Mangifera indica*) had a protective effect against overall oxidation of chicken breast meat during long-term frozen storage for one year. The improved oxidative stability appears to be associated with the inhibition and deceleration of both lipid and protein oxidations, without apparently affecting other meat quality parameters. Further research is needed to optimize the quantity of plant extract mixture in animal diet to maintain its meat quality during chilling storage.

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### **4.3 Influence of pork back fat replacement by hempseed oil emulsion on the oxidative stability of lipids and proteins in cooked sausages**

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## **Abstract**

The present study was aimed to evaluate the influence of partial or total replacement of pork back-fat (60% and 100%) by hempseed oil-in-water emulsion on the chemical composition, oxidative stability, and sensory analysis of reduced fat sausages enriched with n-3 polyunsaturated fatty acids (PUFA). The application of hempseed oil caused a significant reduction of saturated fatty acids (SFA), significant increase in PUFA and significant reduction in the ratio of n-6/n-3. The addition of hempseed oil to sausages caused a significant increase on peroxide value (PV), phytosterol content and phytosterols oxidation products (POPs), but it did not significantly affect thiobarbituric acid-reactive substances (TBARs), cholesterol content, cholesterol oxidation products (COPs) and thiol content. Moreover, protein carbonyls significantly decreased with increasing amount of hempseed oil. Cooked sausages with 60% replacement showed good acceptability scores, similar to those of samples formulated with pork back fat (control). The usage of hempseed oil-in-water emulsion for partial replacement of pork back fat could be a good strategy to improve the nutritional properties of cooked sausages, without impairing their oxidative stability and sensory profile.

*Keywords:* Fat-replacers; Hempseed oil; Cooked sausage; Lipid profile; Lipid oxidation; Protein oxidation.

## 1. Introduction

Meat and meat products are important sources of various nutrients that are essential for growth and development (Jiménez-Colmenero et al., 2012; Santos et al., 2013; Beiloune et al., 2014). Sausages contain up to 30% saturated fatty acid (SFA), which are important for their processing, textural, and sensory characteristics. Besides SFA, sausages are also rich in cholesterol, the two of them deriving from animal fat and thus having a limited place in the nowadays concept of healthy diet (Ospina-E. et al., 2012). In fact, the relatively high amounts of fat, SFA and cholesterol, are risk factors of certain chronic diseases, mainly cardiovascular disease (CVD), cancer, obesity and type 2 diabetes (McAfee et al., 2010; Ferguson, 2010; Corpet et al., 2011; Wyness et al., 2011; McNeill & Van Elswyk, 2012; Pan et al., 2012; Rodríguez-Carpena et al., 2012). Therefore, there is a need to find fat replacers that will permit the development of meat products with a lower content of SFA, *trans* fatty acids, and cholesterol which, at the same time, are able to preserve the sensory qualities and shelf-life of the products (Muguerza et al., 2003; 2004; Sampaio, et al., 2004). Several researchers suggested that the improvement of fat content and quality in meat products is based on reformulation strategies with another fat that is more in line with health recommendations, which include the reduction of total fat (caloric), cholesterol, and modification of the fatty acid profile (i.e. smaller percentages of SFA, larger percentages of monounsaturated fatty acids (MUFA), n-3 PUFA (especially long-chain) or conjugate linoleic acid (CLA) isomers, better n-6/n-3 PUFA and PUFA/SFA ratios) (Wood et al., 2008; Trindade et al., 2011; Berasategi et al., 2011; Jiménez Colmenero et al., 2010, 2012; Olmedilla-Alonso et al., 2013). A variety of vegetable oils (linseed, sunflower, canola, grape seed, avocado, and olive oils) have been added to different meat products as partial substitutes pork back fat (Jiménez- Colmenero et al., 2010; Rodríguez-Carpena et al., 2011,

2012; Berasategi et al., 2011, 2014; Poyato et al., 2014, 2015; Lee et al., 2015). The incorporation of plant oils can be achieved by direct addition of liquid oil, encapsulation and pre-emulsion of oil. The use of intermediate processes, such as pre-emulsions using different types of proteins, represents an improved way of incorporating oil in meat matrices, being able to use higher levels of fat substitution than with the direct use of oils (Muguerza et al., 2001; 2002; 2003; 2004; Martin et al., 2008; Ansorena & Astiasaran, 2004; Valencia et al., 2006; Pelsler et al., 2007; Yildiz-Turp & Serdaroglu, 2008; Choi et al., 2009, 2010). In fact, it is possible to develop Bologna-type sausages with very low amounts of pork back-fat using different emulsifiers (Sanjeeva et al., 2010; Omana et al., 2012; Poyato et al., 2015).

Several studies have demonstrated that the substitution of pork back-fat with PUFA emulsified oils is a good strategy to achieve healthier lipid profiles in these meat products (García-Iniguez de Ciriano et al., 2010; Berasategi et al., 2011; Rodríguez-Carpena et al., 2012). An important factor to consider for the reformulation of meat products is the n-6/n-3 PUFA and the PUFA/SFA ratios which, according to the nutritional recommendations, should be below 4 and greater than 0.4, respectively (Simopoulos, 2006). However, the replacement of SFA by unsaturated fatty acids (PUFA or MUFA) when using vegetable oil instead of animal fat, could lead to an increase of lipid oxidation, which might cause sensory problems and the generation of potentially toxic compounds. Moreover, lipid oxidation products can interact with proteins, and promote their oxidation, with consequent modifications in their structural properties, which can affect the product texture (Estévez, 2011). Therefore, to develop new stable meat products rich in n-3 PUFA, plant oils should be encapsulated or incorporated as emulsion, in combination with suitable antioxidants, to improve their protection.

Hempseed oil represents an interesting alternative to produce new meat products, since it is an exceptionally rich source of PUFAs ( $\leq 80\%$  of the total oil content) (Kiralan et al., 2010; Da Porto et al. 2012; Chen et al., 2010; Teh & Birch 2013) with a n-6/n-3 ratio of 3:1 that matches human optimal nutritional needs according to the European Food Safety Agency recommendations (3-5:1, EFSA, 2009). Moreover, this oil contains linoleic and  $\alpha$ -linolenic acids as its major n-6 and n-3 fatty acids, respectively, together with significant amounts of gamma-linolenic (C18:3n-6) and stearidonic (C18:4n-3) acids (Petrovic et al., 2015). Thus, the replacement of pork back-fat with hempseed oil could be a potentially good strategy to improve health-related traits of cooked sausages, without disregarding the oxidative stability and sensory quality of the products.

The aim of this study was to study the effect of the partial or total replacement of pork back fat by hempseed oil emulsion on the oxidative stability of lipids and proteins in cooked sausages. Chemical characterization of the products was performed, as well as the hedonic evaluation of their sensory characteristics.

## **2. Materials and methods**

### *2.1 Reagents and solvents*

All chemicals used were of analytical grade. Ammonium thiocyanate ( $\text{NH}_4\text{SCN}$ ,  $\geq 97.5\%$ ), barium chloride dihydrate ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), iron (II) sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), sodium dihydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ,  $\geq 99.0\%$ ), tri-sodium phosphate ( $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), diethyl ether, pyridine, hexamethyldisilazane, trimethylchlorosilane and double distilled water, were supplied by Carlo Erba (Milan, Italy). Chloroform, methanol, ethanol, hydrochloric acid (HCl, 37%), potassium chloride (KCl), *n*-

hexane and isopropanol, were purchased from Merck (Darmstadt, Germany). The standard mixture of fatty acid methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN, USA). 1,1,3,3-tetraethoxypropane (precursor for malondialdehyde (MDA)), trichloroacetic acid (TCA,  $\text{CCl}_3\text{COOH}$ ,  $\geq 99.0\%$ ), 2-thiobarbituric acid ( $\geq 98.0\%$ ), anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ,  $\geq 99.0\%$ ), sodium pyrophosphate ( $\text{Na}_4\text{O}_7\text{P}_2$ ,  $\geq 95.0\%$ ), trizma maleate ( $\geq 99.5\%$ ), magnesium chloride ( $\text{MgCl}_2$ ,  $\geq 98.0\%$ ), ethylene glycol-bis(2-aminoethylether)-tetraacetic acid (EGTA  $\geq 97.0\%$ ), 2,4-dinitrophenylhydrazine (DNPH,  $\geq 97.0\%$ ), bovine album serum (BSA,  $\geq 96\%$ ), guanidine hydrochloride ( $\geq 99.0\%$ ), potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ,  $\geq 99.0\%$ ), ethyl acetate ( $\geq 99.7\%$ ), tris(hydroxymethyl)-aminomethane (TRIS,  $\geq 99.8\%$ ), sodium dodecyl sulfate (SDS,  $\geq 99.0\%$ ), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB,  $\geq 98.0\%$ ) and L-cysteine ( $\geq 98.5\%$ ), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Filters papers (90 and 185 mm diameter) were used (Whatman, Maidstone, England). Aminopropyl solid-phase extraction (SPE) cartridges (Strata, 500 mg/3 mL) (Phenomenex Torrence, CA, USA), were utilized for sterol oxides purification. The silylation mixture was prepared with dried pyridine, hexamethyldisilazane and trimethylchlorosilane at a ratio of 5:2:1 by volume.

## *2.2 Materials for sausage formulation*

Pork meat and back fat were obtained from a local meat market (Navarra, Spain). Cold-pressed hempseed oil was supplied by Biolasi Productos Naturales (Guipúzcoa, Spain). BDRom Carne (a mixture of typical aromatic compounds) and the red colorant Carmin de Cochenille 50% (E-120) were obtained from BDF Natural Ingredients S.L. (Girona, Spain). Curavi (a mixture of curing agents: NaCl, E-250, E-252 and antioxidant E-331) was kindly donated by ANVISA (Arganda del Rey, Madrid, Spain).



### 2.3 Sausage formulation and processing

Three batches of bologna-type sausages were manufactured in a pilot plant: Control, 60% replacement, 100% replacement. The total amount of each batch was 7 kg. Table 1 shows all ingredients of the control batch and the two modified batches (60% replacement, 100% replacement). Hempseed oil emulsion was prepared as described in Valencia et al. (2007). The emulsion was prepared by mixing nine parts of water with one part of isolated solid soy protein, and then with ten parts of hempseed oil. Diverse emulsion percentages were mixed with pork fat according to the replacement: 60% (6% pork fat + 9% emulsion) and 100% replacement (0% pork fat + 15% emulsion).

**Table 1.** Formulation of the three types of bologna sausages.

Ingredients	Control	60% replacement	100% replacement
Pork meat (%)	55	55	55
Pork back fat (%)	15	6	0
Hemp oil emulsion (%)	0	9	15
Ice (%)	30	30	30
Iodized NaCl (g/kg)	182	182	182
Milk powdered (g/kg)	84	84	84
Garlic (g/kg)	21	21	21
Curavi <sup>a</sup> (g/kg)	21	21	21
Polyphosphates <sup>b</sup> (g/kg)	14	14	14
Sodium ascorbate (g/kg)	3.5	3.5	3.5
BHT <sup>c</sup> (g/kg)	1.4	1.4	1.4
BDRom carne <sup>d</sup> (g/kg)	7	7	7
Monosodium glutamate (g/kg)	7	7	7
Carmin de Cochenille 50%(E-120) (g/kg)	0.7	0.7	0.7

<sup>a</sup> Curavi: a mixture of curing agents: NaCl, E-250, E-252 and antioxidant E-331.

<sup>b</sup> Mixture of E-430i, E-454i and E-451i.

<sup>c</sup> BHT, butyl hydroxytoluene.

<sup>d</sup> BDRom Carne, commercial aromas mixture.

All ingredients were thoroughly minced in a chilled cutter for 1 min at low speed and 2 min at high speed until a complete emulsification of the mixture was obtained. After the application of a vacuum process to exclude oxygen from the mixture for 2 min, the batters were stuffed in 6-cm

diameter, water impermeable plastic casings. Sausages were cooked in a water bath at 80 °C for 1 h, until the core of the product reached 72 °C. Once heating was completed, the sausages were immediately cooled in a water bath for 2 h and stored frozen (−20 °C) under vacuum till analysis. The experiment was run in triplicate.

### **3. Analytical procedures**

#### *3.1.1 Proximal analysis*

Moisture, protein and ash content were analyzed according to the Association of Official Analytical Chemists (AOAC) official methods (AOAC, 2002a, 2002b, 2002c).

#### *3.1.2 Lipid extraction*

Lipids were extracted according to Boselli et al. (2005), a modified version of the method suggested by Folch, Lees, and Sloane-Stanley (1957). The frozen samples were minced, a subsample of 15 g was taken, and homogenized with 200 mL of a chloroform:methanol solution (1:1, v/v) in a glass bottle with screw-cap. The bottle was kept in an oven at 60 °C for 20 min before adding 100 mL chloroform. After 3 min of homogenization, the mixture was filtered through filter paper; the filtrate was mixed thoroughly with 100 mL of a 1 M KCl solution and left overnight at 4 °C to allow phase separation. The lipid-containing phase (lower) was collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Two lipid extractions were performed per each sample.

#### *3.2 Fatty acid composition*

About 20 mg of lipid extract were methylated with 200  $\mu$ L of diazomethane (Fieser & Fieser, 1967); 1.11 mg of tridecanoic acid methyl ester was added (as internal standard), and the mixture was transmethylated with 40  $\mu$ L of 2 N KOH in methanol (European Commission, 2002), vortexed for 1 min, kept for 5 min, and centrifuged at 1620  $\times$  g for 5 min. Supernatant was transferred to a vial before being injected into a gas chromatograph coupled to a flame ionization detector (GC-FID). The GC-FID instrument was a GC8000 series (Fisons Instruments, Milan, Italy) coupled to a computerized system for data acquisition (Chromcard Data System, ver. 2.3.1, Fisons Instruments). A Rtx-2330 (90% biscyanopropyl/10% phenylcyanopropyl-polysiloxane) fused-silica column (105 m  $\times$  0.25 mm  $\times$  0.2  $\mu$ m film thickness) (Bellefonte, USA), was used. Oven temperature was programmed from 100  $^{\circ}$ C to 180  $^{\circ}$ C at a rate of 3  $^{\circ}$ C/min, kept at 180  $^{\circ}$ C for 10 min, and then taken to 240  $^{\circ}$ C at a rate of 3  $^{\circ}$ C/min; the final temperature was kept for 30 min. The injector and detector temperatures were both set at 250  $^{\circ}$ C. Helium was used as carrier gas at a constant pressure of 260 kPa. The split ratio was 1:50. Peak identification was carried out by comparing the peak retention times with those of the GLC 463 FAME standard mixture. The GC response factor of each fatty acid was calculated by using the GLC 463 FAME standard mixture and the internal standard (13:0). The limit of detection (LOD) of FAMES was 0.0024 mg, whereas the limit of quantification (LOQ) was 0.008 mg. LOD and LOQ were calculated as signal-to-noise ratios equal to 3:1 and 10:1, respectively. Three replicates were analyzed per sample.

### *3.3 Peroxide Value (PV)*

Peroxide value was determined using a modified version of the method of Shantha and Decker (1994). In this method, peroxides oxidize ferrous ions to ferric ions, which react with

ammonium thiocyanate to give rise to a colored complex that can be measured spectrophotometrically. Briefly, 20 mg of extracted lipids were mixed with 9.8 mL of chloroform:methanol (2:1, v/v) and 50  $\mu$ L of thiocyanate/ $\text{Fe}^{2+}$  solution, and then vortexed. After 5 min, the absorbance was measured at 500 nm using a double beam UV-VIS spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan). PV was calculated using a Fe (III) standard calibration curve with a concentration range of 0.599-47.95  $\mu\text{g}/\text{mL}$  ( $y = 0.028 + 0.019x$ ;  $r^2 = 0.996$ ). PV was expressed as meq of  $\text{O}_2$  per kg of fat. Two replicates were run per sample.

#### *3.4 Thiobarbituric acid reactive substances (TBARs)*

TBARs were used to evaluate secondary lipid oxidation products according to the modified method of Witte et al. (1970). This method is based on the reaction between the thiobarbituric acid with aldehydes deriving from secondary oxidation of lipids present in meat, resulting in a colored complex that can be measured spectrophotometrically. Briefly, 8 mL of phosphate buffer aqueous solution at pH 7 were added to 2 g of meat in a 25 mL Sovirel tube and the resulting mixture was homogenized using an Ultra-Turrax T 25 BASIC (Ika-Werke, Staufen, Germany). Two mL of a 30% (v/v) TCA aqueous solution was then added to the sample mixture, homogenized, and filtered. Five mL of 0.02 M aqueous solution of TBA was added to 5 mL of the resulting sample solution in capped tubes, which were kept at 90  $^{\circ}\text{C}$  for 20 min and then maintained at 4  $^{\circ}\text{C}$  for 30 min. After centrifugation, the absorbance of the supernatant was measured at 530 nm with a UV spectrophotometer (Jasco model V-550, Jasco International, Tokyo, Japan). For the quantitative determination of TBARs, a 1,1,3,3-tetramethoxypropane standard calibration curve was used with a concentration range of 1.89-141.50  $\times 10^{-10}$  mol/mL ( $y$

= 0.0072 + 0.0077 x;  $r^2 = 0.999$ ). The TBARs value was expressed as mg of malondialdehyde (MDA)/kg of sample. Two replicates were run per sample.

### *3.5 Determination of sterols*

About 200 mg of extracted lipids were added with known amounts of the internal standard solution (0.140 mg of betulinol and 0.0125 mg of 19-hydroxycholesterol for the determination of total sterols and COPs, respectively). Subsequently, the sample was dried under nitrogen and treated with 10 mL of 1 N KOH solution in methanol to perform a saponification at room temperature for 18 h (Sander et al., 1989). For the extraction of the unsaponifiable matter, 10 mL of water and 10 mL of diethyl ether were added to the samples, which were shaken, and the diethyl ether fraction was separated; the extraction with 10 mL of diethyl ether was repeated twice. The three portions of diethyl ether were pooled, treated with 5 mL of a 0.5 N KOH solution, and extracted. The resulting ethereal extract was washed twice with 5 mL of water. After elimination of excess water by addition of anhydrous sodium sulfate, the ether solution was finally evaporated in a rotary evaporator. The unsaponifiable matter was then diluted in 1 mL of *n*-hexane:isopropanol (4:1, v/v). One-tenth of the unsaponifiable matter was used for the determination of total sterols, whereas the remaining part was utilized for COP analysis.

### *3.6 Fast GC/MS analysis and quantification of cholesterol and phytosterols*

About 500  $\mu$ L of the unsaponifiable fraction were silylated (Sweeley et al., 1963), dried under nitrogen flow and dissolved in 150  $\mu$ L of *n*-hexane. Then, 1  $\mu$ L of sample was injected in split mode (1:50). Fast GC/MS analysis was performed using a Shimadzu QP 2010 Plus GC (Kyoto, Japan) equipped with a split-splitless injector and coupled to a EI mass spectrometric

detector. A fused silica capillary column Restek RTX-5 (10 m x 0.1 mm i.d. x 0.1  $\mu\text{m}$  film thickness; Bellafonte, PA, USA) coated with 95% dimethyl- and 5% diphenyl-polysiloxane, was used. The temperature was programmed from 240 to 325  $^{\circ}\text{C}$  at 5  $^{\circ}\text{C}/\text{min}$ , then increased until 350  $^{\circ}\text{C}$  at 30  $^{\circ}\text{C}/\text{min}$  and finally held at 350  $^{\circ}\text{C}$  for 7 min. The injector temperature was set at 340  $^{\circ}\text{C}$ , the ion source at 200  $^{\circ}\text{C}$  and the transfer line at 340  $^{\circ}\text{C}$ . Helium was used as the carrier gas and linear velocity was 47.7 cm/s. The electron energy was 70 eV. A mass range from  $m/z$  40 to 650 was scanned at a rate of 1500 amu/s. Acquisition and integration modes were full-scan total ion current (TIC) and single ion monitoring (SIM), respectively. The corresponding target ion of cholesterol ( $m/z$  368),  $\beta$ -sitosterol ( $m/z$  396), stigmasterol ( $m/z$  83) and campesterol ( $m/z$  459) were used for their identification. Sterol quantification was carried out in SIM mode with betulinol ( $m/z$  496) as internal standard. The quantification of sterol amount was performed by using calibration curves built for each chemical compounds. The sterol concentration range of the calibration curves was 5–500  $\mu\text{g}/\text{mL}$  ( $r^2 = 0.999$ ).

### *3.7 SOPs purification and Fast GC/MS analysis and quantification*

The remainder of the unsaponifiable fraction (9/10) was dried under nitrogen flow and dissolved in 800  $\mu\text{L}$  of *n*-hexane:ethyl acetate (95:5, v/v) and purified by SPE-NH<sub>2</sub> according to Rose-Sallin et al. (1995). The purified fraction was silylated, dried and dissolved in 50  $\mu\text{L}$  of *n*-hexane. Then, 1  $\mu\text{L}$  of sample was injected in split mode (1:30) for Fast GC/MS analysis that was performed by using the same column described for sterol analysis. The temperature was programmed from 250 to 350  $^{\circ}\text{C}$  at 20  $^{\circ}\text{C}/\text{min}$ . The injector temperature was set at 340  $^{\circ}\text{C}$  and the ion source temperature was set at 200  $^{\circ}\text{C}$ . Helium was used as the carrier gas and linear velocity was 43.0 cm/s. Helium inlet pressure was 426.7 kPa. The acquisition and integration

modes were Full Scan (TIC) and Single Ion Monitoring (SIM), respectively. Cholesterol oxidation products (COPs) and phytosterol oxidation products (POPs) were identified according to their reference ions (Cardenia et al., 2012; Leal-Castañeda et al., 2015). The following target ions were used to quantify the COPs:  $m/z$  353 (19-OH);  $m/z$  456 ( $7\alpha$ -OH and  $7\beta$ -OH);  $m/z$  384 ( $5,6\alpha$ -E and  $5,6\beta$ -E);  $m/z$  403 (CT);  $m/z$  131 (25-OH) and  $m/z$  472 (7-K). Quantification was performed using the respective calibration curves in SIM mode, plotting the ratio of COP and IS areas as function of the ratio of COP and IS concentrations. The COPs concentration range of the calibration curves was 0.01–0.6  $\mu\text{g/mL}$  ( $r^2 = 0.99$ – $1.00$ ). Considering that most POPs standards are not commercially available and assuming that POP fragmentation is similar to that of COPs, the quantification of POPs was carried out using calibration curves of oxysterols built by quantifier ions with relative abundances similar to those of the ions chosen for oxysterol quantification.

### *3.8 Protein carbonyl content*

Protein carbonyl groups were evaluated by derivatization with DNPH, as described by Levine et al. (1990). One g of sample was minced and homogenized in 10 mL of pyrophosphate buffer solution (pH 7.4) that contained 2 mM  $\text{Na}_4\text{O}_7\text{P}_2$ , 10 mM tris-maleate, 2 mM  $\text{MgCl}_2$ , 100 mM KCl, and 2 mM EGTA, by using Ultra Turrax at 12,000 rpm for 60 s. Two equal volumes of meat homogenate (0.1 mL) were precipitated with 1 mL of 20% TCA and centrifuged at 14,000  $\times$  g for 5 min at room temperature, and the supernatant was removed. For the determination of carbonyl content, 1 mL of 2 M HCl containing 0.2% DNPH was added, while for protein quantification 1 mL of 2 M HCl was added. Both samples were incubated for 1 h at room temperature in the dark and vortexed every 20 min. After incubation, 1 mL of 20% TCA was

added to each tube and vortex for 30 s, then were centrifuged at 14,000 x g for 5 min and the supernatant removed. DNPH was removed by washing the pellet with 1 mL of ethanol:ethyl acetate (1:1, v/v) mixture, shaken and centrifuged for 5 min at 14,000 x g. This step was repeated three times. The pellet was finally solubilized in 2 mL of 6.0 M guanidine hydrochloride dissolved in 20 mM potassium phosphate monobasic (pH 2.3). The samples were kept at 4 °C overnight, and the final solution was centrifuged at 14,000 x g for 15 min to remove insoluble materials. Protein concentration was determined by measuring the absorbance at 280 nm in HCl treated samples against a standard calibration curve of BSA dissolved in 6 M guanidine HCl, with a concentration range of 0.2-1.0 mg/mL ( $y = 1.956 + 0.007x$ ;  $r^2 = 0.997$ ). Absorbance at 370 nm of the DNPH samples was measured. The carbonyl content in nmol/mg protein was calculated as described by Levine et al. (1994), using an absorption coefficient at 370 nm of 22,000/M cm for the formed hydrazones. Three replicates were run per sample.

### *3.9 Protein thiol content*

Sulphydryl groups (thiol content) were determined according to Nieto et al. (2013). The concentration of thiol groups in meat samples was quantified after derivatization with DTNB as suggested by Ellman (1959). One g of meat was homogenized in 25 mL of 5.0% SDS dissolved in 100 mM TRIS buffer (pH 8.0) using an Ultra Turrax at 8000 rpm for 30 sec. The homogenates were placed in a water bath at 80 °C for 30 min. After cooling, they were centrifuged at 14000 ×g for 10 min. The supernatant was diluted to a concentration of 1.5 mg/mL with the buffer used for homogenization (0.5 mL of the homogenate was diluted with 2.0 mL 5% SDS in 0.1 M TRIS buffer at pH 8.0). The dilution was analyzed according to Liu and Xiong (2000) by mixing 500 μL sample, 2mL of 100 mM TRIS buffer (pH 8.0), and 500 μL of 10 mM DTNB dissolved in



100 mM TRIS buffer (pH 8.0). The absorbance at 412 nm was measured before addition of DTNB ( $ABS_{412\text{-before}}$ ) and after reaction with DTNB ( $ABS_{412\text{-after}}$ ) against a reference solution of 500  $\mu\text{L}$  5% SDS in 100 mM TRIS buffer (pH 8.0) and 2.50 mL of 100 mM TRIS buffer (pH 8.0). The mixture was allowed to react protected against light for exactly 30 min. A solution containing 2mL 100 mM TRIS buffer (pH 8.0), 500  $\mu\text{L}$  5% SDS and 500  $\mu\text{L}$  10 mM DTNB was used as blank sample ( $ABS_{412\text{-blank}}$ ). The protein concentration was determined spectrophotometrically at 280 nm using a standard curve prepared from BSA dissolved in 5.0% SDS in 100 mM TRIS buffer (pH 8.0) with a concentration range of 0.3-3.0 mg/mL ( $y = 1.645 + 0.007x$ ;  $r^2 = 0.999$ ). The corrected absorbance was determined by:  $\text{Abs change} = ABS_{412\text{-after}} - ABS_{412\text{-before}} - ABS_{412\text{-blank}}$ . The final thiol concentration was calculated standard calibration curve prepared from L-cysteine diluted in 5.0% SDS in 100 mM TRIS buffer (pH 8.0), with a concentration range of 5-200  $\mu\text{M}$  thiols ( $y = 583.2 + 26.65x$ ;  $r^2 = 0.993$ ). The thiol content was calculated in nmol thiol/mg protein. Three replicates were run per sample.

### *3.10 Sensory analysis*

Sensory analysis was carried out to compare the sensory characteristics of the control and modified sausages (60% and 100%). Forty non-trained panelists were given three samples in a single session; 50 g of each sample were put in small plastic dishes identified with a random three-digit code and served a balanced complete block experimental design. Panelists were asked to score modified batches on a 5-point scale in which the degree of acceptability for different attributes was evaluated: color, flavor, texture and overall acceptability. A value of 1 corresponded to “very poor” for each attribute and a value of 5 to “very good”. Control samples were taken as the reference value, receiving a score of 5 points for every attribute.

#### 4. Statistical analysis

The data are reported as mean values of three independent replicates (n=3) for each treatment group (total n=15). Tukey's honest significance test was performed at a 95% confidence level ( $p \leq 0.05$ ), in order to separate means of statistically difference. Statistical analysis of the data was performed by SPSS 20.0.0 (2011, IBM-SPSS Inc., Chicago, Illinois, USA).

#### 5. Results and discussion

##### 5.1 Proximate composition of the formulated sausages

The study was designed to obtain a formulation with the minimum pork back fat content and maximum water content, maintaining adequate technological and sensory properties. Significant differences were detected in fat content of the three products (control, 60% and 100% replacement). The replacement of pork back fat with hempseed oil significantly decreased ( $p < 0.05$ ) the amount of total lipids in the final product for control, 60% and 100% replacement (14.99, 12.01 and 10.04 g/100 g, respectively) (Table 2).

**Table 2.** Chemical composition of the three formulations.

Formulation	Control			60%			100%		Stat. Signif.	
	Mean	SE		Mean	SE		Mean	SE		
Moisture %	69.77	0.34	b	73.13	0.14	a	72.88	0.37	a	0.007
Protein %	12.71	0.11		13.31	0.02		14.08	0.42		ns
Fat %	14.99	0.21	a	12.01	0.16	b	10.04	0.15	c	0.001
Ash %	2.93	0.52		2.74	0.71		3.52	0.04		ns

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

No statistical differences were found for the amounts of protein (12.71–14.08 g/100 g) and ash (2.74–3.52 g/100 g) among the different formulations with increasing water content (69.77 g/100 g in control, 72.88–73.13 g/100 g in 60% and 100% replacement, respectively) (Table 2); these data are in agreement with Poyato et al. (2014), who used linseed oil as fat replacer in the formulation of cooked meat products.

### *5.2 Fatty acids profile of hempseed oil*

As in most vegetable oils, SFA (9.85%) had the smallest share in hempseed oil, with palmitic and stearic acids as the most predominant SFA. The MUFA content was slightly higher (11.63%), being oleic acid the most abundant. PUFA are the predominant fatty acid class in hempseed oil, amounting to 78.53% of total fatty acid. The essential fatty acid linoleic acid (LA, 56.02%) and alpha-linolenic acid (ALA, 17.66%), made up most of the PUFA content. Gamma-linolenic acid (3.74%) and stearidonic acid (1.07%) were also detected, which are characteristic of hempseed oil and rarely present in vegetable oils. The FA composition of the hempseed oil used in this study is similar to those reported by Chen et al. (2010), and Teh and Birch (2013). The average ratio of PUFA: MUFA: SFA was 78.53:11.63:9.85, which is in good agreement with other studies (Kiralan et al., 2010; Da Porto et al., 2012).

### *5.3 Fatty acid composition of the formulated sausages*

Table 3 shows the fatty acid composition of different sausages formulations manufactured with hempseed oil as replacers of pork back fat. As expected, the treatment with hempseed oil had a significant effect on the fatty acid composition of these products; other researchers (Bersategi et al., 2011, 2014; Poyato et al., 2014, 2015) also found substantial changes in the

lipid composition of different meat products when replacing animal fat by vegetable oils. The addition of hempseed oil significantly affected SFA ( $p < 0.05$ ), reflecting its particular composition which is in agreement with Ansorena and Astiasarán (2004) when they used vegetables oils as pork back fat replacer. Five fatty acids namely palmitic, stearic, oleic, linoleic acids and  $\alpha$ -linolenic acid comprised around 90% of total fatty acids analyzed in cooked sausages. The fatty acid profiles among treated formulations were different as they reflected the specific fatty acid composition of hempseed oil employed for the product manufacture. The reduction in SFA in products with hempseed oil increased the percentage of PUFA from 22.44% in control to 41.52% and 61.76% in the 60% and 100% substitution of pork back fat, respectively. Among the three formulations of cooked sausages, control had the highest percentage of oleic acid (36.74%), followed by the 60% replacement (28.04%) and 100% replacement (18.37%). The 100% replacement had the largest proportion of linoleic acid (45.01%), followed by the 60% replacement (31.97%) and the control (19.97%).

Regarding nutritional aspects, SFA increase the low density lipoproteins (LDL), and according to Rodríguez-Carpena et al. (2012), the intake of SFA could lead to an increase in cholesterol levels in the blood. The nutritional quality of the lipid fraction of food can be evaluated through the PUFA/SFA ratio. Increases in this ratio could lead to a reduction in total cholesterol in the blood plasma (McAfee et al., 2010). The products formulated with hempseed oil significantly increased ( $p < 0.05$ ) the PUFA/SFA ratio with respect to products prepared with pork back fat. When reducing and replacing the pork back fat with hempseed oil, the aim was to reduce SFA and, at the same time, to increase PUFA, especially n-3 ones (Ospina-E. et al., 2011). The most relevant changes were observed for the n-6/n-3 ratio between control (15.08) and 60% and 100% replacement (4.52–3.49, respectively), the latter being in agreement with the current nutritional

recommendation for this ratio (4) (Simopoulos, 2006). In fact, the n-6/n-3 ratio significantly decreased with the addition of hempseed oil to the formulation.

**Table 3.** Fatty acid composition of the three types of bologna-type sausages (expressed as g/100 g of lipids).

Fatty acids	Control			60% replacement			100% replacement			Stat. Signif.
	Mean	SE		Mean	SE		Mean	SE		
C10:0	0.07	0.00	a	0.05	0.00	b	0.02	0.00	c	0.001
C12:0	0.07	0.00	a	0.05	0.00	b	0.03	0.00	c	0.001
C14:0	1.03	0.02	a	0.64	0.02	b	0.29	0.01	c	0.001
C15:0	0.06	0.00	a	0.04	0.00	b	0.02	0.00	c	0.001
C16:0	16.64	0.18	a	12.41	0.18	b	8.42	0.05	c	0.001
C16:1	1.61	0.03	a	1.13	0.03	b	0.58	0.01	c	0.001
C17:0	0.26	0.01	a	0.17	0.01	b	0.09	0.00	c	0.001
C18:0	7.99	0.05	a	5.92	0.05	b	3.93	0.04	c	0.001
C18:1 (n-9)	29.86	0.21	a	22.64	0.21	b	15.15	0.23	c	0.001
C18:1 (n-7)	2.32	0.03	a	1.78	0.03	b	1.25	0.02	c	0.001
C18:2 (n-6)	16.23	0.33	c	25.82	0.58	b	37.11	0.29	a	0.001
C18:3 (n-6)	0.16	0.01	c	1.22	0.08	b	2.30	0.04	a	0.001
C18:3 (n-3)	1.02	0.03	c	5.68	0.19	b	10.60	0.12	a	0.001
C20:1 (n-9)	0.57	0.03	a	0.46	0.01	b	0.38	0.01	c	0.001
C18:4(n-3)	0.00	0.00	c	0.33	0.02	b	0.70	0.01	a	0.001
C20:2 (n-6)	0.61	0.04	a	0.35	0.00	b	0.16	0.00	c	0.001
C20:3 (n-3)	0.13	0.01	a	0.09	0.00	b	0.06	0.00	c	0.001
C22:1	0.52	0.03	a	0.42	0.02	b	0.41	0.00	b	0.013
C22:4 (n-6)	0.11	0.01	a	0.07	0.00	b	0.06	0.00	b	0.001
C22:5 (n-3)	0.12	0.01	a	0.08	0.01	b	0.06	0.00	b	0.001
Σ SFA	26.10	0.15	a	19.28	0.20	b	12.80	0.10	c	0.001
Σ UFA	53.26	1.17	c	59.76	1.12	b	68.11	0.56	a	0.001
Σ MUFA	34.89	0.84	a	26.11	0.27	b	17.07	0.25	c	0.001
Σ PUFA	18.38	0.42	c	33.65	0.86	b	51.04	0.45	a	0.001
Σ PUFA/Σ SFA	0.70	0.00	c	1.73	0.03	b	4.03	0.07	a	0.001
ΣUFA/Σ SFA	2.04	0.05	c	3.10	0.03	b	5.33	0.08	a	0.001
n-6	17.23	0.39	c	27.56	0.66	b	39.68	0.32	a	0.001
n-3	1.14	0.03	c	6.09	0.21	b	11.36	0.13	a	0.001
n-6/ n-3	15.08	0.19	a	4.52	0.05	b	3.49	0.01	c	0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

Since n-6 and n-3 fatty acids cannot be synthesized by humans, PUFA content in meat products are very important from the nutritional standpoint. The principal dietary source of n-6 is linoleic acid, which is found at high concentrations in pork unlike in other meat species (Wood et al.,

2003). The amount of ALA in control products was 0.27 g/100 g, whereas in modified products it varied between 2.31 and 2.57 g/100 g. However, it has been demonstrated that the increased consumption of ALA has no beneficial effect on health (Wang et al., 2006). ALA is a nutritionally essential one required for synthesis of important long-chain n-3 PUFA and eicosanoids. Dietary n-3 fatty acids play a key role in terms of inflammatory reduction, HDL increase and LDL reduction (Johnston, 2009). Poyato et al. (2014) observed that the replacement of pork back-fat by 50% gelled-oil-in-water emulsion increased n-3 PUFA in bologna sausage which induces low level of oxidation.

#### *5.4 Sterols content*

Table 4 shows the effects of pork back fat replacement on the single and total sterol contents of different cooked sausages. Total sterol content ranged from 3577 mg/kg lipids (control) to 6808 mg/kg lipids (100% replacement), which corresponded to 53 and 68 mg sterols/100 g meat, respectively; the total sterol content was significantly higher ( $p < 0.05$ ) in the 100% replacement with respect to the control one. Among sterols, total cholesterol content of the cooked sausages ranged from 3577 to 4007 mg/kg lipids (~40–53 mg/100 g product) in the 100% replacement and control samples, respectively, it must be noted that the registered variation in the cholesterol content was due to reduced fat content of different formulations of sausages.

Total cholesterol was about 100% of total sterols for control sample while it constituted about 74% and 59% of total sterols for the 60% and 100% replacement samples. When the presence of hempseed oil in the cooked sausage formulation was increased, a significant ( $p < 0.05$ ) rise of the total sterols and total and single phytosterol content was noticed, where  $\beta$ -sitosterol was the most

abundant (78-81% of phytosterols), followed by campesterol (15-16%) and stigmasterol (4%). Plant sterols are known to have several bioactive properties with possible implications for human health, such as blood cholesterol lowering capacity; in fact, they are able to lower total and LDL blood cholesterol by preventing cholesterol absorption from the intestine, with a 10-12.5% decreased absorption if consumed 3 g/day (EFSA, 2012). Therefore, incorporation of phytosterols into the cooked sausage would somehow enhance its biofunctionality.

**Table 4.** Sterols contents of the three formulations of bologna sausages.

	Control		60% replacement		100% replacement		Stat. Signif.			
	Mean	SE	Mean	SE	Mean	SE				
mg/100 mg fat										
Cholesterol	3577.58	62.47	3832.74	294.06	4007.58	231.30	0.430			
Stigmasterol	nd	0.00	c	54.10	5.76	b	107.83	6.76	a	0.001
Sitostanol	nd	0.00	b	nd	0.00	b	42.88	3.30	a	0.001
Campesterol	nd	0.00	c	198.00	8.17	b	456.87	11.74	a	0.001
β-Sitosterol	nd	0.00	c	1083.80	27.24	b	2193.08	25.14	a	0.001
Total Sterols	3577.58	62.47	c	5168.62	318.49	b	6808.24	249.62	a	0.001
mg/100 g product	Mean	SE		Mean	SE		Mean	SE		
Cholesterol	53.63	0.94	a	46.03	3.53	ab	40.24	2.32	b	0.025
Stigmasterol	nd	0.00	c	0.65	0.07	b	1.08	0.07	a	0.001
Sitostanol	nd	0.00	b	nd	0.00	b	0.43	0.03	a	0.001
Campesterol	nd	0.00	c	2.38	0.10	b	4.59	0.12	a	0.001
β-Sitosterol	nd	0.00	c	13.02	0.33	b	22.02	0.25	a	0.001
Total Sterols	53.63	0.94	b	62.08	3.83	ab	68.36	2.51	a	0.023

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

In accordance with the present results, other authors found a significant decrease of cholesterol in meat products as a result of a partial substitution of animal fat with vegetable oil, such as olive and sunflower oils (Choi et al., 2010; Lee et al., 2015; Poyato et al., 2015). Compared to the sausage from the present study, the meat products analyzed in those papers had considerably higher fat contents and the replacement of animal fat was also combined with a reduction of the total amount of animal fat. On the other hand, Muguera et al. (2003) reported that replacing up

to 25% of pork back-fat with vegetable oils had no impact on the cholesterol levels of fermented sausages containing around 33% fat. Rodríguez-Carpena et al. (2012) reported that replacing of 50% of pork back-fat with vegetable oils had no impact on the cholesterol levels in emulsified pork burger patties containing around 14% fat.

### *5.5 Cholesterol oxidation products (COPs)*

Table 5 shows the effects of pork back fat replacement on the single and total COP contents of different cooked sausages samples, as well as on their cholesterol oxidation rates. Besides proving useful information about the extent of cholesterol oxidation in food products of animal or mixed origin, there is an interest in monitoring COPs as they are well-known for their role on the onset and development of major chronic diseases (such as neurodegenerative processes, atherosclerosis, diabetes, osteoporosis and kidney failure), together with a series of negative biological effects (pro-inflammatory, pro-apoptotic, cytotoxic, carcinogenic and mutagenic) (Otaegui-Arrazola et al., 2010).

Total COP content of the cooked sausages ranged from 9.0 to 14.7 mg/kg of lipids (~0.14–0.15 mg/100 g product). Eder et al. (2005) found a linear correlation between soybean oil concentration and oxysterol content in sausage obtained from pig that had been fed with soybean oil. COPs levels found in control and reformulated cooked sausages (136-148 µg/100 g of meat) were above the threshold of toxicological concern (TTC) for unclassified compounds (0.15 µg per person per day) (Kroes et al., 2004), so they might represent a potential risk for human health and thus further research is needed required to better ascertain their toxicity levels.

COPs did not significantly ( $p > 0.05$ ) changed between different treatments, but an increasing trend on COPs formation was noted with increasing amount of hempseed oil in the reformulated



cooked sausages. The most abundant COPs were  $\beta$ -epoxycholesterol ( $\beta$ -CE, 30% of total COPs), followed by  $\alpha$ -epoxycholesterol ( $\alpha$ -CE, 22%), 7-ketocholesterol (7-KC, 16%),  $7\beta$ -hydroxycholesterol ( $7\beta$ -HC, 12%) and  $7\alpha$ -hydroxycholesterol ( $7\alpha$ -HC, 10%) and triol (10%) in all treatments.

**Table 5.** Content of cholesterol oxidation products (COPs) of the three formulations of bologna sausages.

	Control			60% replacement			100% replacement			Stat. Signif.
	Mean	SE		Mean	SE		Mean	SE		
$\mu\text{g}/100\text{ mg fat}$										
7 $\alpha$ -hydroxycholesterol	0.09	0.00	c	0.11	0.00	b	0.13	0.00	a	0.001
7 $\beta$ -hydroxycholesterol	0.11	0.01	c	0.14	0.01	b	0.17	0.01	a	0.001
$\alpha$ -CE	0.20	0.00	c	0.26	0.01	b	0.32	0.02	a	0.001
$\beta$ -CE	0.28	0.01	c	0.36	0.01	b	0.46	0.01	a	0.001
Triol	0.09	0.00	c	0.12	0.01	b	0.15	0.01	a	0.001
7-ketocholesterol	0.15	0.01	c	0.20	0.00	b	0.24	0.01	a	0.001
Total COPs	0.90	0.03	c	1.18	0.02	b	1.47	0.03	a	0.001
Ratio COPs %	0.25	0.01	b	0.31	0.03	ab	0.37	0.03	a	0.035
$\mu\text{g}/100\text{ g product}$	Mean	SE		Mean	SE		Mean	SE		
7 $\alpha$ -hydroxycholesterol	13.03	0.39		13.14	0.13		13.33	0.15		0.693
7 $\beta$ -hydroxycholesterol	16.60	0.75		16.52	0.70		16.79	0.46		0.953
$\alpha$ -CE	29.28	0.53		30.69	0.86		32.06	1.99		0.376
$\beta$ -CE	41.14	1.97		43.73	1.11		46.24	1.38		0.140
Triol	13.65	0.61		14.62	0.62		15.33	0.76		0.282
7-ketocholesterol	21.92	1.14		23.55	0.44		24.15	0.77		0.230
Total COPs	135.63	4.01		142.25	2.87		147.91	2.70		0.095

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

Apparently, the formulation and cooking conditions used for these meat products favored the bimolecular oxidation pathway of cholesterol, leading to the formation of the epoxy derivatives that constituted more than 50% of total COPs; following the same oxidation pathway and in presence of water (69-73%) and acidic conditions, the epoxy-ring opened with the consequent generation of triol (10% of total COPs), which is considered one of the most toxic COPs. The other 38% of COPs was constituted by 7-oxysterols, of which 7-KC was the predominant one; this compound is often used as maker of cholesterol oxidation due to its fast formation and

accumulation but, as confirmed by these data, 7-KC is not always the most abundant COP in cooked meat products as other cholesterol oxidation pathways can be favored and/or 7-KC can react with other matrices compounds, such as proteins.

The cholesterol oxidation ratio (%OR, calculated as % COPs/cholesterol) varied from 0.25% to 0.37%, being significantly higher in the 100% replacement sample with respect to the control one; this confirms the increasing trend on COPs formation observed with increasing amount of hempseed oil in the reformulated cooked sausages.

### *5.6 Phytosterols oxidation products (POPs)*

Table 6 shows the effects of pork back fat replacement on the single and total POPs contents of different sausages samples, as well as on their phytosterol oxidation rates. Total POP content of the cooked sausages ranged from 7.6 to 12.6 mg/kg of lipids (~0.09–0.13 mg/100 g product). To the best of our knowledge, there are no data available in literature about POPs content in meat products reformulated with vegetable oils. The results are in agreement with data reported by Menéndez-Carreño et al. (2016), who detected 0.5-9.7 mg POP/100 g of cooked/baked meat when using phytosterol-enriched margarine. POPs increased with increasing amount of hempseed oil in the reformulated cooked sausages with 60% and 100% fat replacement, which confirms the importance of the unsaturation degree of FA (59.8% and 68.1% unsaturated fatty acids of which 33.7% and 51.0% PUFA, respectively) as oxidation promoting factor. Only  $\beta$ -sitosterol oxides were found in the two reformulated products, which showed significant differences ( $p < 0.05$ ) in the contents of all single POPs (except the triol derivative) and the total POPs. However, sitotrienol was the most abundant POP (27-33% of total POPs), followed by 7-ketositosterol (20-23%) and 7 $\alpha$ -hydroxysitosterol (15-18%), 6-ketositosterol (15-

18%) and 7 $\beta$ -hydroxysitosterol (13-16%). Most  $\beta$ -sitosterol oxides (about 70%) derived from the monomolecular pathway of 7-hydroperoxide decomposition, with 7-ketositosterol as the 2o. most abundant POP. It must be noted that no epoxy derivatives were detected and that the reaction equilibrium of the bimolecular oxidation pathway of  $\beta$ -sitosterol moved towards triol formation.

The phytosterol oxidation ratio (%OR, calculated as % POPs/phytosterols) varied from not detected to 0.32%, showing a significant ( $p < 0.05$ ) increase with increasing amount of hempseed oil in the reformulated cooked sausages.

**Table 6.** Content of phytosterols oxidation products (POPs) of the three formulations of bologna sausages.

	Control			60% replacement			100% replacement		Stat. Signif.	
	Mean	SE		Mean	SE		Mean	SE		
$\mu\text{g}/100 \text{ mg fat}$										
7 $\alpha$ -Hydroxysitosterol	nd	0.00	c	0.12	0.00	b	0.23	0.00	a	0.001
7 $\beta$ -hydroxysitosterol	Nd	0.00	c	0.10	0.00	b	0.20	0.02	a	0.001
7-Ketositosterol	nd	0.00	c	0.16	0.02	b	0.27	0.02	a	0.001
6-ketositosterol	nd	0.00	c	0.12	0.01	b	0.22	0.01	a	0.001
Sitotrienol	nd	0.00	b	0.26	0.03	a	0.34	0.06	a	0.001
Total POPs	nd	0.00	c	0.76	0.04	b	1.26	0.10	a	0.001
Ratio POPs %	nd	0.00	c	0.20	0.01	b	0.32	0.04	a	0.001
$\mu\text{g}/100\text{g product}$	Mean	SE		Mean	SE		Mean	SE		
7 $\alpha$ -Hydroxysitosterol	nd	0.00	c	14.43	0.52	b	23.18	0.42	a	0.001
7 $\beta$ -hydroxysitosterol	nd	0.00	c	12.45	0.05	b	20.23	1.56	a	0.001
7-Ketositosterol	nd	0.00	c	18.75	1.99	b	27.49	2.11	a	0.001
6-ketositosterol	nd	0.00	c	13.83	1.14	b	21.79	0.67	a	0.001
Sitotrienol	nd	0.00	b	31.46	3.42	a	33.78	5.56	a	0.001
Total POPs	nd	0.00	c	90.92	4.99	b	126.46	9.51	a	0.001

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ).

Qualitative and quantitative differences in COPs and POPs formation could be due to the different starting amount of sterols, as well as the diverse physical location of cholesterol and phytosterol in the meat products. In fact, while cholesterol was mainly present in the cell

membranes of the muscle tissues, the vegetable oil containing phytosterols was introduced as an oil-in water (O/W) emulsion into the product. Phytosterols are known to migrate at the interface of the emulsion droplets where oxidative stress is high, thereby being more susceptible to oxidation in (O/W) emulsion than in bulk oil (Cercaci et al., 2007).

### *5.7 Lipid oxidation*

Table 7 reports the PV and TBARs data of the three cooked sausage formulations. PV varied from 0.56 to 1.34 meq O<sub>2</sub>/kg and it significantly rose ( $p < 0.05$ ) with increasing amount of PUFA. As previously discussed, the reformulated samples had a higher FA unsaturation degree, which reflected in a higher level of primary oxidation. In any case, PV of all samples were low and far below the threshold of 20 meq of O<sub>2</sub>/kg of lipids associated with oil rancidity (Ripoll et al., 2011). It is possible that, besides the added BHT as antioxidant, the naturally occurring antioxidants in hempseed oil might have contributed to the oxidative stability of the meat product, despite the high PUFA level; in fact, fresh cold-pressed hempseed oil shows a high antioxidant activity, which has been associated to the presence of phenolic compounds, especially flavonoids (such as flavanones, flavonols, flavanols and isoflavones) (Smeriglio et al., 2016), as well as tocopherols ( $\gamma$ -tocopherol  $\gg$   $\alpha$ -tocopherol) (Kriese et al., 2004; Teh & Birch, 2013). The amount of antioxidant present in hempseed oil is highly related to its variety and freshness (Kriese et al., 2004; Teh & Birch, 2013).

Regarding TBARs, they varied from 0.38 to 0.44 mg MDA/kg and no significant differences ( $p > 0.05$ ) were observed among samples. TBARs of all samples were below 0.5 mg MDA/kg, which is considered the critical limit for rancid odor and taste perception by consumers (Wood et al., 2008). These results are in agreement with Muguerza et al. (2003) and Rodriguez-carpena et

al. (2011). Moreover, when referring to emulsified meat products, most of the existent researches reported TBARs lower than 1 mg MDA/kg sample (Ansorena & Astiasaran, 2004; Yıldız-Turp & Serdaroglu, 2008; Choi et al., 2010). Cáceres et al. (2008) found TBARs values of 0.37 mg MDA/kg in “mortadellas” prepared with fish oil, and they did not find differences in TBARs values of control and fish oil added sausages.

**Table 7.** Peroxide value (PV, meq O<sub>2</sub>/kg fat), TBARs (mg MDA/kg meat), carbonyl content (nm carbonyl/mg protein), and thiol content (nm thiol/mg protein) of the three types of bologna-type sausages.

Formulation	Control		c	60%		b	100%		a	Stat. Signif.
	Mean	SE		Mean	SE		Mean	SE		
PV	0.56	0.04		1.03	0.05		1.34	0.07		0.001
TBARs	0.38	0.02		0.41	0.02		0.44	0.02		ns
Carbonyl	11.30	0.35	a	10.31	0.28	ab	9.45	0.15	b	0.008
Thiol	48.10	0.95		47.57	0.93		47.40	0.76		ns

Results are expressed as mean and SE, standard error of the three independent samples (n=3). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey’s test;  $p \leq 0.05$ ).

However, Bersategi et al. (2014) and Poyato et al. (2015) reported that Bologna-type sausages and burger patties formulated with vegetable oils had lower TBARs numbers than those formulated with animal fat. As aforementioned, BHT was added to minimize PUFA's oxidation, but the natural antioxidants contained in the hempseed oil (mainly phenolic compounds) might have contributed to improve the overall oxidative stability of modified sausages through the inhibition of TBARs formation. It is also possible that, during cooking, part of the aldehydes have reacted with proteins, forming Schiff bases. The results from previous works on this issue evidence conflicting effects of vegetable oils when used as fat replacers in meat products. In agreement with the present results, some authors have reported significantly lower amounts of lipid oxidation products in muscle foods produced with olive and high-oleic sunflower oil than in

the control counterparts (Muguerza et al., 2001; Ansorena & Astiasarán, 2004). In contrast, results from other studies support that using vegetable oils for the manufacture of muscle foods enhances the oxidative instability of the final product (Kayaardi & Gök, 2003; Choi et al., 2010). The hempseed oil employed in the present study as replacer of animal back fat did not promote oxidation of modified sausages. This is in agreement with Berasategi et al. (2011, 2014) and Poyato et al. (2014), who used linseed oil as animal fat replacer in a meat product (Bologna type sausages).

### *5.8 Protein oxidation*

Protein oxidation in the three types of sausages was evaluated by the formation of protein carbonyls and loss of protein thiols (Table 7). Carbonyls varied from 9.45 to 11.30 nm carbonyl/mg protein and it significantly decreased ( $p < 0.05$ ) with increasing amount of hempseed oil, especially when comparing 100% replacement and control samples. This protective effect is dependent on the composition of the raw materials, in particular the type and concentration of antioxidants. In meat systems, the formation of carbonyl compounds is a well-establish consequence of the metal catalyzed oxidation of certain amino acids, such as lysine, arginine and proline (Estévez, 2011). In this case, bioactive phenols present in hempseed oil could have acted as metal quenchers (Smeriglio et al. (2016), while tocopherols could have protected proteins against ROS as these compounds have been proven to be reliable inhibitors of myofibrillar protein oxidation (Estévez et al., 2008b).

Formation of carbonyls is one of the most prominent changes in oxidized proteins, and the content of carbonyl is widely used as a marker of protein damage (Lund et al., 2008). Oxidative reactions can also occur in the side chains of amino acids and give rise to the formation of

carbonyl groups (Xiong, 2000). The occurrence of protein oxidation in muscle foods is thought to take place along with lipid oxidation. In fact, numerous meat researchers have reported a timely interaction between lipid and protein oxidation (Estévez et al., 2008a; Ventanas et al., 2007), which is supported by the fact that ROS formed during early stages of PUFA oxidation can attack susceptible amino acid residues to trigger their oxidative degradation (Lund et al., 2011). Consequently, the extent of protein oxidation in meat products has been reported to be enhanced with increasing amounts of lipids (Estévez et al., 2005). Protein thiols varied from 47.40 to 48.10 nm thiols/mg protein and despite the addition of hempseed oil tended to reduce the thiol concentration, but the decrease was not significant compared to the control sausages. The values slightly higher than levels found in Bologna type sausages prepared from oxidatively stressed pork (Lund et al., 2008) and in agreement with levels found in oxidized fresh beef (Jongberg et al., 2011).

The thiol group of cysteine (RSH) is highly susceptible to oxidation in the presence of hydrogen peroxide, which is formed in cells and accumulated in meat post-mortem (Lund et al., 2011). Primary and secondary lipid oxidation products can act as reactants for protein oxidation, so once the oxidation of lipids starts, the oxidation of proteins will also occur but at diverse reaction kinetics (Estevez et al., 2011). Modification of the amino acid side chain has been reported to also result in the formation of thiol groups (Stadtman, 1990). The derivatives of interactions between lipid oxidation products and amino acid residues can cause formation of cross-linkage between proteins (Zamora et al., 2000). The cross-linked protein has been described as the formation of disulfide and dityrosine through the loss of cysteine and tyrosine residues (Estevez et al., 2009).

### 5.9 Sensory evaluation

Considering that sensory acceptability is a crucial factor when testing the incorporation of new ingredients in reformulated food products, a hedonic test was performed in all samples in order to evaluate consumer acceptability of the reformulated cooked sausages. Samples were subjected to multiple comparison tests between control and the modified products (control, 60% and 100%) (Table 8). The number of correct answers was determined and data corresponded to the mean value obtained for each type of product by the 40 panelists. Results showed significant differences ( $p < 0.05$ ) for color, flavor, texture, and overall acceptance index between the modified product with 100% replacement and the control. On the contrary, the product with 60% replacement gave statistically similar results to the control one. In summary, these results led to conclude that the product formulated with 60% of pork back fat replacement was satisfactory for the panelist, so that the hempseed oil ingredient could be a good fat replacer for obtaining sausages where no sensory problems could be detected. Similarly, Gao et al. (2014) reported improvements or no significant differences in meat products in which partial fat replacements were performed.

**Table 8.** Scores of hedonic sensory analysis, control and the different level of substitution (60%, 100%) are given a 5 point score for every attribute.

	Control			60%			100%		Stat. Signif.	
	Mean	SE		Mean	SE		Mean	SE		
Colour	3.73	0.15	a	3.58	0.15	a	3.23	0.17	b	0.001
Texture	3.30	0.18	a	3.23	0.20	a	2.70	0.20	b	0.001
Flavor	3.43	0.19	a	3.33	0.17	a	2.90	0.20	b	0.001
Overall acceptance Index	3.40	0.17	a	3.23	0.20	a	2.83	0.20	b	0.001

Results are expressed as mean and SE, standard error of the three independent samples (A total of 40 panelist participated). Abbreviations: Control, 15% pork back fat; 60% replacement (6% pork back fat and 9% hemp emulsion); 100% replacement (0% pork back fat and 15% hemp emulsion); Stat. signif., statistical significance; a, b, c, statistically different means (Tukey's test;  $p \leq 0.05$ ). Scores: 1. very poor; 2. poor; 3. standard; 4. good; 5. Very good.



## **6. Conclusions**

Addition of hempseed oil-in-water emulsion for partial and total replacement of pork back-fat in cooked sausages reformulation, significantly modified the fatty acids composition, leading to a reduction of SFA and to an, improved n-6/n-3 ratio. The addition of hempseed oil to sausages caused a significant increase on PV and POPs, but the other parameters of lipid and protein oxidation were not significantly affected. In addition, cooked sausages with 60% replacement had good acceptability scores, similar to those of samples formulated with pork back fat (control). In conclusion, the usage of hempseed oil-in-water emulsion for partial replacement of pork back fat could be a good strategy to improve the nutritional properties of cooked sausages, without impairing their oxidative stability and sensory profile.

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## 5. Conclusions

The general objective of this PhD thesis was to study the effects of dietary supplementation, product formulation and storage conditions on the oxidative stability of lipids and proteins in meat and meat products, with the final aim to better understand the trends of these oxidative processes in such products in order to identify strategies to improve their overall quality.

In the first study, photooxidation of both lipid and protein fractions in raw meat stored under commercial retail conditions, was investigated. Beef and chicken meat slices were packed in vessels with transparent shrink film and exposed to white fluorescent light 4 °C for 0, 3 and 5 days (12-h light/12-h darkness cycle). Lipid oxidation was assessed by measuring peroxide value (PV) and thiobarbituric acid reactive substances (TBARs), while the quantification of thiol and carbonyl groups were used to evaluate the extent of protein oxidation. In general, both lipid and protein oxidation parameters significantly increased during storage at 4 °C under darkness conditions, with a greater impact when exposed to light. In general, beef meat FA composition was partially ( $p < 0.05$ ) affected by storage conditions and exposure to light, while no significant effects ( $p > 0.05$ ) were observed in the FA composition of chicken breast meat. After 5 days of chill storage of beef meat, exposure to light led to a significant decrease ( $p < 0.05$ ) on n-3 FA, as well as to a significant increase ( $p < 0.05$ ) on both  $\sum\text{SFA}/\sum\text{UFA}$  and n-6/n-3 FA ratios. Beef meat exhibited much higher lipid and protein oxidation (about 12 and 2 times, respectively) than chicken breast. TBARs and protein oxidation parameters were directly correlated, thus suggesting an interaction between both oxidative processes. This study evidenced that storage time and exposure to light can greatly affect both lipid and protein oxidations in raw meats,

especially beef meat, thus suitable strategies (i.e. film types and/or active packaging) should be found to improve their oxidative stability and reduce the impact of light exposure.

The second experiment focused on the effect of plant extracts mixture on the oxidative stability of chicken breast meat during chill and frozen storage. The experiment was set up according to a Randomized Complete Block Design, with 2 experimental groups and 12 replications per treatment. Male Ross 308 broilers were fed either a diet supplemented with or without plant extract mixture (0.35 g/kg feed) for 42 days. Freshly prepared samples were stored for short-term storage at 4 °C for 0 and 7 days, then packed in plastic bags under vacuum and subjected to long-term frozen storage at -18 °C for one year. No effect of the antioxidant plant extract mixture on lipid oxidation of fresh meat samples stored at 4 °C, was observed. After frozen storage at -18 °C for 1 year, fatty acid composition was significantly ( $p < 0.05$ ) modified in both types of samples, but they were differently affected; in particular, the antioxidant dietary treatment led to a higher preservation of n-3 PUFA. Moreover, samples obtained with dietary antioxidant supplementation had significantly lower PV, TBARs and carbonyls contents and higher values of sulfhydryl level than the basal diet meat. In the long-term stored samples, lipid oxidation parameters were directly correlated with those of protein oxidation, confirming the interaction between both oxidative processes. These results suggest that dietary antioxidants can minimize the oxidative stability of both lipids and proteins in chicken breast meat during long-term frozen storage.

The third experiment aimed at evaluating the influence of partial or total replacement of pork back-fat (60% and 100%) by hempseed oil-in-water emulsion on the chemical composition, oxidative stability, and sensory analysis of reduced fat sausages enriched with n-3 polyunsaturated fatty acids (PUFA). The application of hempseed oil caused a significant

reduction of saturated fatty acids, a significant increase in PUFA and a significant decrease in the n-6/n-3 ratio, leading to a value below or close to the current nutritional recommendation for the latter ratio (4). Addition of hempseed oil to sausages caused a significant increase on PV, phytosterols and their oxidation products, but it did not significantly affect TBARs, thiol content, cholesterol and its oxidation products. Moreover, protein carbonyls significantly decreased with increasing amount of hempseed oil. Cooked sausages with 60% replacement showed good acceptability scores, similar to those of samples formulated with pork back fat (control). The usage of hempseed oil-in-water emulsion for partial replacement of pork back fat could be a good strategy to improve the nutritional properties of cooked sausages, without impairing their oxidative stability and sensory profile.



## **6. Dissemination of results**

### **Publications:**

Zaazaa A., Savioli S., Cardenia V., Rodriguez-Estrada M.T. Photooxidation of lipids and proteins of chicken and beef raw meats during storage under commercial retail conditions. To be sent to *Meat Science* journal.

Zaazaa A., Ansorena D., Astiasarán I., Cardenia V., Inchingolo R., Rodriguez-Estrada M.T. Influence of pork back fat replacement by hempseed oil emulsion on the oxidative stability of lipid and protein in cooked sausages. To be sent to *Meat Science* journal.

Zaazaa A., Sirri F., Cardenia V., Rodriguez-Estrada M.T. Effect of plant extracts mixture on the oxidative stability of chicken breast meat during chill and frozen storage. To be sent to *Meat Science* journal.

### **Poster communication in Congress and Conferences:**

Zaazaa A. (2016). The effects of dietary supplementation and product formulation on the oxidative stability of lipids and proteins in meat and meat products, Proceedings of the "Giornata del Dottorato – Curriculum in Scienze e Biotecnologie degli Alimenti", 1<sup>o</sup> Aprile 2016 (Cesena, Italy).

Rodriguez-Estrada M.T., Zaazaa A., Savioli S., Cardenia V. (2015). Photooxidation of lipids and proteins of different types of raw meats during storage under commercial retail conditions. Book of abstracts of the 13<sup>th</sup> Euro Fed Lipid Congress. pp. 290.

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