Alma Mater Studiorum

Università di Bologna

DOTTORATO DI RICERCA

SCIENZE E BIOTECNOLOGIE DEGLI ALIMENTI

Ciclo XXV

Settore Concorsuale di afferenza: 07 F1

Settore Scientifico disciplinare: AGR 15

TITOLO TESI

Lipid quality and oxidative stability in food products from animal origin, as affected by breeding factor

Presentata da: Dott.ssa Antonietta Funaro

Coordinatore Dottorato

Prof. Claudio Cavani

Relatore

Dott.ssa Maria Teresa Rodriguez Estrada

Correlatore

Prof. Claudio Cavani

Esame finale anno 2013

CONTENTS

ABSTRACT

Nowadays it is requested more investigations on alternative rearing systems that are able to improve poultry welfare and to warrant high-quality and safe meat products. This thesis work was focused on the evaluation of the oxidative stability of poultry meats, obtained with different rearing systems, diets (supplemented with bioactive compounds), and packaging conditions.

The thesis work was divided into the following parts:

- Evaluation of the effects of different rearing systems on the quality, fatty acid composition and oxidative stability of poultry thigh and breast meat belonging to different product categories ("rotisserie" and "cut-up" carcasses);
- Evaluation of the effects of different rearing systems and packaging conditions on the shelf-life of poultry thigh meat stored at 4°C for 14 days, and the effects of feed supplementation with thymol (control diet and diet with 2 different concentration of thymol) and packaging conditions on lipid oxidation of poultry thigh meat shelf-life (stored at 4°C for 14 days). The oxidative stability of poultry meat was studied by means of the spectrophotometric determinations of peroxide value and thiobarbituric acid reactive substances.
- Evaluation of anti-inflammatory effects of different flavonoids (thymol, luteolin, tangeretin, sulforaphane, polymethoxyflavones, curcumin derivates) to detect their biological activity in LPS-stimulated RAW 264.7 macrophage cells *in vitro*, in order to study more in depth their action mechanisms. It was evaluated the cell vitality (MTT assay), nitrite concentration and protein profile. The study was focused on the identification of potential dietary bioactive compounds in order to investigate their biological activity and possible synergic effects, and to develop new suitable strategies for long-term promotion of human health, in particular against cancer.

Keywords: poultry meat, lipid oxidation, rearing systems, packaging, shelf-life, bioflavonoids, inflammation

DEDICATION

With all my love to my Dears

ACKNOWLEDGEMENTS

A special thanks to Dr. Maria Teresa Rodriguez Estrada, to Professor Claudio Cavani, to Dr. Massimiliano Petracci and to Dr. Vladimiro Cardenia

1 Review of the literature

1.1 Meat quality, animal welfare and environmental protection

Over the past few years, the concept of food has undergone a radical transformation, as it does not have only sensory and nutritional properties, but it also plays an important role on human health status and on the reduction of the onset of many diseases. Therefore, the modern nutritional science has changed the meaning of the classical concepts of "suitable" nutrition to "positive" or "optimal" diet, because health problems associated with overnutrition dominate all over the world.

Scientific research and the communication of the findings are of the extreme importance in consumer education. Indeed, the lifestyle and nutrition choices that individuals make influence the obesity rates (Schonfeldt et al., 2008). However, the interest of Food Science research is to improve the complex food system, making it safe, tasty, nutritious and giving more information about food quality and safety to each consumer. The vast food system includes agricultural production and harvesting, holding and storing of raw materials, food manufacturing (formulation, food processing, and packaging), transportation and distribution. Contemporary food science and technology contributed greatly to the success of this modern food system by integrating several disciplines to face nutritional deficiencies and enhancing food safety (Floros et al., 2010).

This led to the identification and development of new research activities called "science of wellness", such as nutrigenomic, whose main objective is the protection of consumer health. In rich societies, consumers increasingly give importance to all those aspects that improve their quality of life. Diet is not the only factor that affects wellbeing and health, but it is one of the most important ones. Factors that have fostered this development include the incredible current need of population to have safer and even healthier food

products. These products must possess one of the following characteristics: modified composition and/or processing conditions to prevent or limit the presence of certain potentially harmful compounds, and/or the possibility of including certain desirable substances, either naturally or by addition, with the subsequent added benefits to health (Jimènez-Colmenero et al., 2001). Growing concerns about public health risks have heightened consumer awareness on food safety issues, after incidents involving microbiological contamination (such as Salmonella), bovine spongiform encephalopathy (BSE), chemical residues in food (such as dioxins) and the possible consequences of technological interventions (such as genetically modified organisms). Understanding consumer perception of risk and impact on purchase behavior is a key issue for the mutual benefit of both consumers and food industry (Yeung et al., 2001). The 'meat quality' concept is multi-factorial and it includes many complementary attributes. The criteria that consumers associate with the meat quality are nutritional value, freshness, leanness, taste, juiciness, healthiness and tenderness besides safety, and convenience (Grunert et al., 2004). Indeed, besides meat safety, nutritional and sensorial properties, and added value level, consumers at the moment wish to have more information about the origin and method of production of meat based food, process characteristics, environmental concern, and animal welfare.

Another element that contributes to the complexity of meat sector is the market indicators: on one side, there is a strong impulse towards more and more competitive production systems due to the globalization process while, on the other side, there is a strong demand to move back towards more extensive production systems, traditional productions, and organic products (Trichopoulou et al., 2007). Livestock and poultry producers face a number of challenges including pressure from the public to be good environmental promoter and adopt welfare-friendly practices (Siegford et al., 2008).

Sometimes environmental encouragement and animal welfare may have conflicting objectives. For instance, many organic standards for food product certification have goals aimed at promoting environmental health and animal welfare (USDA, 2007).

The consumer demand for environmentally friendly, chemical free and healthy products, as well as concern regarding industrial agriculture's effect on the environment, has led to a significant growth of organic farming. Consumers, producers, and even government organizations are becoming more interested in the ecological footprints left by animal-based agriculture and the consequences for animal and human welfare (Siegford et al., 2008). Systems could be designed to take advantage of the natural behavior of animals in such a way that they promote animal productivity, as well as the other crop productivity incorporated into the systems (Hermansen et al., 2004). Ultimately, analysis and research at the interface between the environment and animal welfare are now needed to determine the environmental vs. welfare costs through the lifecycle of animal-based products to gain a better understanding of the environmental costs of ethical animal production (Siegford et al., 2008).

In poultry sectors, most meat products that reach the food market are produced using animals reared under intensive conditions. Animals belong to genotypes that have been selected and crossed for rapid growth and feed efficiency and are kept indoors in stables equipped with strict environmental control (photoperiod, light intensity, temperature, relative humidity, etc.), (Fletcher, 2002). However, following the multiple and growing demand of consumers, who are more sensitive to the ethical and cultural aspects of food consumption, there is an increasing interest in Europe towards alternative rearing and animal-friendly production systems, which can improve animal welfare as well as guarantee higher qualitative standards of food safety, nutritional, and sensory properties (Magdelaine et al., 2008).

In poultry, recent studies performed under alternative housing systems evidenced that lower stocking densities increased possibility of movement both in indoor housing and in outdoor areas and different vegetable feed sources in open-air runs, may contribute to modify the product quality (Castellini et al., 2008). Indeed, birds kept in alternative housing systems show lower carcass fat depots and higher polyunsaturated fatty acids (PUFA) content that enhance the nutritional value of meat but reduce its oxidative stability (López-Bote et al., 1998; Ponte et al., 2008). Moreover, the use of slow growing breeds and strains and, consequently, the longer rearing period, can affect meat tenderness and sensory traits, as well as its acceptability by consumers (Farmer et al., 1997; Fanatico et al., 2005).

1.2 The role of meat on human health: risks and benefits associated with consumption of meat

According to a worldwide accepted definition, meat is all edible parts of a carcass of a slaughtered animal (EU, 2004). This includes the lean muscular tissue, adjacent (intra- and intermuscular) fat, adipose tissue, usually called fatty tissue, and also includes blood, organs (offal), and intestines.

It is very important to consider that muscle tissue is a complex biological structure consisted of epimysium (connective tissue surrounding the entire muscle), perimysium (connective tissue separating the groups of fibers into bundles), and endomysia (sheaths of connective tissue surrounding each muscle fiber). Each muscle fiber is surrounded of the sarcolemma membrane, which periodically, along the length of the fiber, forms invaginations usually referred to as T tubules. The sarcoplasm serves to suspend organelles such as mitochondria and lysosomes and it contains 75-80% of water plus lipid droplets, variable quantities of glycogen granules, ribosomes, proteins,

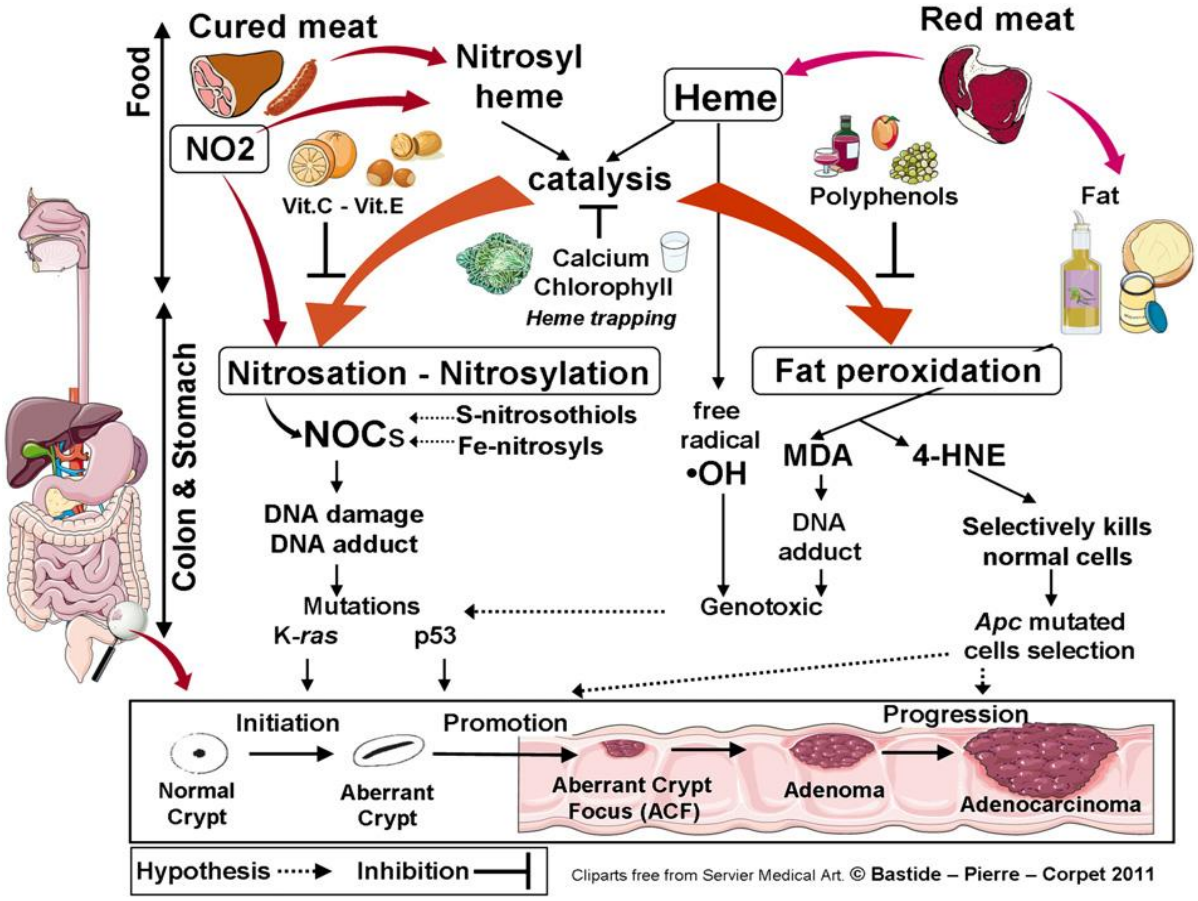
nonprotein nitrogenous compounds, and a number of inorganic constituents (Erickson, 2002).

The main constituent of all meat and poultry products is water, which varies from 30 to 70%. During manufacturing of thermally-treated meat products, water (20–25%) is often added for technical and sensory reasons. Other important constituent of muscle foods, unprocessed or processed, are proteins, whose content vary among the different meat cuts and meat products. Protein content in meat and poultry, including their processed products, ranges between about 10 and 23%. Another macronutrient is fat, which may be as low as 1%, but varies up to around 50% in some dried raw sausages (Honikel, 2008).

Meat has an important place in a healthy diet, as it provides balanced proteins, essential amino acids, fat, iron high bioavailability, vitamins (especially those of the B group) and other essential minerals (such as zinc) (Wood et al., 2007; Williamson et al., 2005; Givens et al., 2006). The significant amount of fat in meat and type of fatty acids (FA) (mainly saturated ones) that constitute it are controversial components that have been most under the spotlight for several years in relation to the healthiness of people that consumes meat (Wood et al., 2007).

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 et al., 2004). Recent meta-analyses show that high consumers of cured meats and red meat are at increased risk of colorectal cancer (Corpet et al., 2011). Heme group catalyzes the formation of apparent total N-nitroso compounds (ATNC) and lipid peroxidation endproducts, which partially explains the promoting effect of red and processed meat on colorectal cancer (CRC) (Figure 1).

Figure 1: Catalytic effect of heme iron on fat peroxidation and N-nitrosation, and their inhibition by dietary means. Consequences for the development of colorectal cancer (from Bastide et al., 2011).



ATNC collectively comprise nitrosyl iron and S-nitrosothiols, besides nitrosamines and nitrosamides (Bastide et al., 2011).

One of the 10 universal guidelines for healthy nutrition reported by the World Cancer Research Fund (WCRF) at the end of 2007, is to "limit intake of red meat and avoid processed meat", as a result of the convincing evidence for an association between processed meats intake and colorectal cancer development (Demeyer et al., 2008). Evidences from human studies also suggest that increased dietary intake of heme iron is associated with increased incidences of colon cancer (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. 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 (Shay et al., 2012). Micha et al. (2012) recently studied the effects of unprocessed (fresh/frozen) red and processed (using sodium/other preservatives) meat consumption on coronary heart disease (CHD) and diabetes. The overall findings suggest that neither unprocessed red or processed meat consumption is 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 an 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). 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., 2010b). 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 (see paragraph 1.4).

1.3 Poultry meat

Poultry meat is consumed and produced all around the world and, over the last few decades, has increased in popularity in many countries. Among the reasons for this increased consumption, it can be listed the relatively low costs of production, the rapid growth rate of poultry, the high nutritional value of the meat and the introduction of many new further processed products.

The poultry industry has changed dramatically over the past 50 years. In the early 1900s, most poultry in the western world was produced in small flocks, mainly to supply eggs to support small farm units, and the eggs and live birds' were sold in local markets. Today, the poultry industry is highly integrated and managed by a number of large corporations (Barbut, 2001). Chicken production varies around the world and it is concentrated in five major areas: America (43.3% of the world's production), Asia (34.1%), Europe (15.9%), Africa (5.2%) and Oceania (1.4 %) (Food and Agriculture organization of United Nation (FAO, 2012) data from www.thepoultrysite.com, Global Poultry Trends). The worldwide chicken meat production is slowly growing, going from 58.7 to 90.9 million tonnes between 2000 and 2012, respectively (FAO 2012, www.thepoultrysite.com, Global Poultry Trends). In Italy,

poultry meat production is currently around 0.9 million tones and represents 6.2% of the total chicken meat production in Europe. (FAO 2012, www.thepoultrysite.com, Global Poultry Trends). In 2010, the Italian chicken meat production increased by 5.2%, while the Italian export was around 40.9% (www.unionenazionaleavicoltura.it). However, the latest available data of poultry meat production showed that chicken meat exportation by Italy is around 2.15% between European countries, and 0.52% in the world (FAO 2009, www.thepoultrysite.com, Global Poultry Trends).

People's diet in industrialized countries usually contains more animal protein than that in rural areas, mainly because urban people are more prosperous, but also because they generally have access to a wider variety of foods at local markets. In low-income countries, commercially produced chicken meat is well placed to satisfy the demands of a rapidly increasing affluent, middle class who can afford to pay for broiler chickens. Facilities and infrastructure for producing broiler chickens can be established quickly and start soon. Therefore, chicken meat is not only a healthy meat, but it is the cheapest of all livestock meats (http://www.fao.org/docrep/013/al714e/al714e00.pdf).

During last years, the changes in consumer's lifestyle in developed countries have led to a meat market more and more addressed towards easy-handled and processed products ("convenience food"). This trend has been exploited by the poultry industry, which made strong investments in the processing area. Further processed products are demanded due to their convenience, high food safety and quality standards, which stimulate the poultry chains to adopt more strict protocols for the production of raw meat (Fletcher, 2002).

Another aspect that has had a great impact on the improvement of poultry market is the nutritional healthy properties of this type of meat. In fact, poultry meat provides not only high-quality protein, but also important vitamins and minerals. Fat content in poultry meat is relatively low (2.8 g/100 g breast and 13 g/100 g thigh) and with a positive unsaturated FA/SFA ratio,

from a human health point (Barroeta, 2007). Moreover, poultry meat has a higher monounsaturated fatty acid (MUFA) and PUFA contents than other meats. Simopoulos (2000) highlights the role of poultry products in enriching the human diet with n-3 PUFA. Current reviews and papers are very convincing on the positive role of enriched poultry, which can be considered a "functional food" as it can provide also bioactive substances with favorable effects on human health, such as n-3 PUFA enrichment (Betti et al., 2009a, b), vitamins (Barroeta, 2007) antioxidants (Grashorn, 2007), vegetable extracts (Aziza et al., 2010; Leusink et al., 2010; Narciso-Gaytàn et al., 2010a; Velasco et al., 2010; Shirahigue et al., 2010), plant bioactive compounds (Wallace et al., 2010).

1.4 Meat lipids

As already mention, meat is a good source of fat, which provide energy, perform many functions in the body and can positively or negatively influence human health (Mapiye et al., 2012). But, in many countries, meat lipids are a controversial macronutrient for human health, but, at the same time, they contribute to meat quality and nutritional value (Wood et al., 2008).

The total lipid content of muscle (or intramuscular fat, often termed marbling fat) is mainly composed of neutral lipids and plays an important role in the tenderness and juiciness of cooked meat (Wood et al., 2008). Lipids are accumulated in the animal carcass primarily under the skin as a layer in the subcutaneous connective tissue consisting of cells almost entirely filled with fat. This tissue has an insulating and contour-building role and serves as a rich energy store. Furthermore, there is the intramuscular fat that comprises phospholipids (PL) that build predominantly the cell membranes, triacylglycerides (TAG) present mainly in the intramuscular adipocytes (about

80%) and as droplets within the myofibers' cytoplasm (5-20%), as white flecks or streaks between the bundles of muscle fibers within the lean muscle tissue, and cholesterol (Sikorski et al., 2010).

The types and levels of fatty acids (FA) in meat lipids depend on the animal species, breed, age, cut and diet (Kauffman, 2001; Sikorski et al., 2010). Chicken broiler meat is a rich source of oleic (~34-41%), linoleic (~13-24%), palmitic (~22-24%), stearic (~8-13%) and palmitoleic (~4-5%) acids (Kamboh et al., 2013; Souci et al., 2008; Bou et al., 2005). Therefore, monounsaturated FA (MUFA) are the most abundant FA class (~45-50%) in poultry meat, followed SFA (~32-37%) and PUFA (~17-23%) (Bou et al., 2005; Kamboh et al., 2013).

The level of fat tissue in the carcass and meat are important underlying factors, because FA composition changes as fat is deposited. One important consequence of increasing fat deposition is that the proportion of PL is reduced, leading to a decrease in the proportion of the major PUFA, 18:2 n-6 (Wood et al., 2007). The FA composition of meat also has crucial effects on meat quality, for example, fat tissue firmness, color, shelf life, and flavor (Wood et al., 2007).

Dietary strategies is the best way to improve nutritional value of animal food products. Animal metabolism plays an important role on the effectiveness of any dietary supplementation and further meat enrichment (Bou et al., 2009). For instance, the fatty acid composition of non-ruminant monogastric animals, such as chicken, can be readily modified by diet, since fatty acids are deposited unchanged by digestion. In ruminants (polygastric animals), instead, the rumen is a barrier for the direct incorporation of PUFA into meat, as rumen enzymes biohydrogenate fat, thus changing their original composition; however, the effect of grass diets in increasing levels of n-3 PUFA and conjugated linoleic acid (CLA) (FA with beneficial effects on human health) is an interesting area of current research, leading to more

desirable meat products for the consumer (Wood et al., 2007). However, animal food products have been reported to be successfully enriched in several compounds through dietary factors (Barroeta et al., 2007; Bou et al., 2009). Therefore, dietary induced modifications of the meat FA profile could be a very positive purpose for human diet.

1.5 Lipids oxidation in muscle foods

One of the main factors limiting the quality and acceptability of meat and meat products is lipid oxidation, as it can impact the nutritional value, shelf-life and sensory quality (flavor and color). Lipid oxidation in foods is one of the major degradation processes that are responsible for food quality loss. The process of lipid oxidation is initiated when a hydrogen atom is removed from a methylene group in the hydrocarbon chain of a lipid molecule, especially from PUFA (Kanner, 2007). Different internal and external factors can impact the extent of oxidation, such as FA composition, FA degree of unsaturation, content and activity of pro- and antioxidants, irradiation, temperature, oxygen pressure, surface area in contact with oxygen, and water activity (a_w) (Kolakowska, 2002).

The autoxidation of unsaturated lipids contained in oils, fats, and food and the endogenous oxidative degradation of membrane lipids by lipid peroxidation result in the formation of a very complex mixture of lipid hydroperoxides, chain-cleavage products, and polymeric material. The oxidation of unsaturated FA results in significant generation of advanced lipid oxidation endproducts, which are in part cytotoxic and genotoxic compounds (Kanner, 2007). Experimental animal studies and biochemical investigations lend support to the hypothesis that lipid-oxidation products ingested with food or produced endogenously, represent a health risk (Esterbauer, 1993). The undesirable effects of lipid oxidation on food quality are reflected on the

sensory attributes (development of off-odor, off-flavor, discoloration, undesirable texture), nutritional value (loss of PUFA, vitamins, antioxidants and damage of proteins, amino acids, formation of protein radicals, lipid-protein interactions), toxicity (generation of hydroperoxides, aldehydes, epoxides, dimers, oxysterols, *trans* fatty acids, Maillard type products) and technological suitability (decrease of emulsifying activity of protein and protein solubility). However, lipid oxidation also involves some desirable effects, such as bactericidal effects of radicals and development of characteristic flavor compounds of some food products (if not in excess) (Kolakowska, 2002).

The main oxidation substrates are PUFA, with several double bonds that are thermodynamically favored sites for attack by lipid peroxyl radicals (Gardner, 1989). Membrane lipids (like PL) distributed throughout the tissue, has an high reactivity as they are mainly constituted by unsaturated FA, whereas TAG or storage lipids are mostly formed by SFA (German, 1990; Slabyj et al., 1984).

On the other hand, Labuza et al. (1969), showed that free FA (FFA) are more prone to oxidation than esterified FA and TAG. Muscle lipids may be exposed to lipolytic enzymes released from lysosomal organelles, which lead to lipid hydrolysis and thus favor lipid oxidation (Erickson, 2002).

The conversion of muscle to meat is a direct result of the cessation of blood flow and the arresting of many metabolic processes. It means that after death of an animal, all circulation ceases, an event that rapidly brings about important changes in the muscle tissue. Table 1 shows biochemical changes and negative consequences that increase prooxidant factors concentration in postmortem muscle food (Erickson, 2002).

Table 1: Biochemical changes and negative consequences in postmortem muscle food (from Erickson, 2002).

Biochemical changes	Consequence
Decrease in ATP	Loss of energy source needed for reduction of many compounds
Increase in hypoxanthine	Off-flavor produced by ATP degradation
Conversion of hypoxanthine dehydrogenase to xanthine oxidase	Xanthine oxidase can initiate oxidation when molecular oxygen is present in system
Decrease in ascorbate, glutathione	Loss of secondary antioxidants and cofactors
Increase in low molecular weight iron	Initiator of oxidation
Oxidation of myoglobin to porphyrin radical	Can react with hydrogen peroxide to produce ferryl oxene (Fe^{4+}), which can initiate lipid oxidation
Loss of tocopherol	Loss of primary antioxidant
Disintegration of membranes	Could cause hydrolysis of phospholipids, uneven maintenance of ions
Loss of Ca^{2+} sequestration	Increased calcium ion content in aqueous phase causes many inactive processes to become active

Moreover, the potential catalysts of lipid oxidation in muscle foods are nonenzymic and enzymic, as shown in Table 2.

Table 2: Potential catalysts of lipid oxidation in muscle foods (from Erickson, 2002).

Nonenzymic
Transition low molecular weight metal ions
Metmyoglobin–H_2O_2
Porphyrin compounds (sensitizers for the generation of singlet oxygen)
Enzymic
Lipoxygenase
Myeloperoxidase
Membrane enzymic systems that reduce iron

In living tissue, control of oxidation is the best way to prevent the destruction of lipid membranes, proteins and nucleic acids. Several systems exist in skeletal muscle to maintain the physiological balance, which control oxidative reactions suspended by processing operations of muscle foods, such as cooking and salting (Decker et al., 1998). Disruption of that oxidative balance causes a rapid development of rancidity in muscle foods. To avoid oxidative process, during food processing technologies, is essential finding a good balance between prooxidative/antioxidative factors of muscle food products, preserving the natural antioxidant stability and manipulating animal diet with antioxidants supplementation (Table 3).

Table 3: Factors influencing prooxidative/antioxidative balance of muscle food products (from Decker et al., 1998).

Oxidation Substrates	Prooxidants	Antioxidants	
		Endogenous	**Exogenous**
Fatty Acids	Transition metals	α-Tocopherol	Nitrite
Oxygen	Enzymes	Ubiquinone	Synthetic phenolics
	Iron-containing proteins	Carotenoids	Plant phenolics
		Ascorbate	Chelators
		Carnosine/anserine	Ascorbate
		Glutathione	
		Polyamines	
		Uric acid	
		Antioxidant enzymes	

Photooxidation, another important nonenzymic catalyst, involves photosensitizer for the formation of singlet-oxygen, or for direct formation of radicals (Faustman et al., 1990). Experimental evidence support the ability of iron heme proteins (including myoglobin and hemoglobin) to promote lipid peroxidation (Hazell, 1982; Kendrick et al., 1969). These proteins are abundant in muscle tissue and their concentration depends on the animal

species and muscle types. Oxygen transport, storage and activation in aerobic organisms depend on the iron porphyrin moiety in heme proteins. Simply iron species originating from degradation of heme proteins and other sources bind to negatively charged phospholipids in membranes and catalyze the cleavage of preformed lipid hydroperoxides (Carlsen et al., 2005). Both color and flavor are some of the most important aspect that influence consumer's perception and expectation. Intrinsic flavor of meat is due to the production of volatile, odorous, lipid oxidation products (aliphatic aldehydes, ketones and alcohols) during cooking, together with Maillard reaction products (Wood et al., 2003). Moreover, preventing production of off-odors and off-flavors to avoid warmed-over flavor phenomenon or meat flavor deterioration by oxidation lipid, is essential to maintain quality and safety in meats (Pearson et al., 1977).

1.6 Modified atmosphere packaging (MAP) and its effect on the muscle food shelf-life

In the food technology literature there are different definitions of shelf-life: "the period between the manufacture and the retail purchase of a food product, during which time the product is in a state of satisfactory quality in terms of nutritional value, taste, texture and appearance" (The Institute of Food Technologists (IFT) in the United States, 1974). Labuza et al. (1988) gave another meaning to this term: "shelf-life is the duration of that period between the packing of a product and the end of consumer quality as determined by the percentage of consumers who are displeased by the product". On the other hand, the Institute of Food Science and Technology (IFST) in the United Kingdom (1993) defined shelf-life "the period of time during which the food product will remain safe; be certain to retain desired sensory, chemical, physical, microbiological and functional characteristics;

and comply with any label declaration of nutritional data when stored under the recommended conditions" (Robertson, 2009a). Therefore, to guarantee consumer's demand, food packaging is essential, because without packaging the safety and quality of food would be compromised,. Food packaging performs several tasks: it protects food from contamination and spoilage, it makes it easier to transport and store food, and it provides uniform measurement of contents (Robertson, 2009a). For the majority of foods and beverages, quality decreases over time and during the storage. To know which types of changes influence food quality is the first step in developing food packaging that will minimize undesirable changes in quality and maximize the development and maintenance of desirable properties. Information about the main factors that control the rates of these reactions is necessary to minimize the changes occurring in foods during storage, then, while packaged (Robertson, 2009b). Few years ago Van Boekel (2008), described clearly the nature of the deteriorative reactions in foods and the factors that control the rates of these reactions outlining the most important quality-related changes:

- Chemical reactions, mainly due to either oxidation or Maillard reactions.
- Microbial reactions: microorganisms can grow in foods; in the case of fermentation, this is desired, otherwise microbial growth will lead to spoilage and, in the case of pathogens, to unsafe food.
- Biochemical reactions: many foods contain endogenous enzymes that can potentially catalyze reactions leading to quality loss (enzymatic browning, lipolysis, proteolysis, and others). In the case of fermentation, enzymes can be exploited to improve quality.
- Physical reactions: many foods are heterogeneous and contain particles. These particles are unstable, in principle at least, and phenomena such as coalescence, aggregation, and sedimentation lead usually to quality

loss. Also, changes in texture can be considered as physical reactions, though the underlying mechanism may be of a chemical nature.

Deteriorative reactions in muscle foods depend on two different types of factors: intrinsic (water activity, a_w, pH, oxidation-reduction potential (E_h), O_2 content, and product formulation), and extrinsic (temperature, relative humidity (%RH), atmosphere, and light) (Robertson, 2009b).

Many of the chemical reactions that occur in muscle foods can lead to deterioration in food quality (both nutritional and sensory) or to the impairment of food safety. The entity of these chemical reactions depends on intrinsic and extrinsic factors that could be controlled by preservative packaging. Therefore, packaging can play a major role in controlling these factors.

Packaging can act over several factors that promote lipid oxidation (such as light, O_2 concentration, catalysts (i.e. iron, copper, heme pigments and a_w), thus helping extending food shelf-life (Robertson, 2009b).

Overall packaging has been a key element to preserve the quality of foods from a microbiological standpoint. Aseptic packaging relies on isolating the sterilized food inside barrier packaging that has been previously decontaminated. The shelf-life of microbiologically perishable foods greatly depends on some packaging, variables such as gas and water vapor barrier properties, atmosphere modification, and active packaging. These variables affect the microbial flora development in food, the spoilage rate, and time. Fresh meats in grocery store are usually packaged mainly in expanded polystyrene (PS) trays wrapped with a stretchable poly(vinyl chloride) (PVC) film. The inner surfaces of the trays may have a low density polyethylene (LDPE) or PVC coating, and the product is usually placed on a soaker pad to absorb the exudate. The overwrapping film (familiar to consumer and inexpensive), which is usually heat-sealed on the underside of the tray, must

have a high O_2 transmission rate (OTR) if discoloration of red meats is not to be accelerated by low O_2 concentration in areas of contact between the meat and the film (Gill, 2009).

The use of preservative packaging for raw meats have led to major changes in the processing and marketing of such products (Gill et al., 2009). Modified atmosphere packaging (MAP) is an effective technique to preserve perishable chilled foods without resorting to heat processing or chemical preservatives. The preserving effect of MAP derives mainly from the use of CO_2 gas, which inhibits or retards microbial growth significantly above a concentration of 20% (Lee, 2009). However, as many studies have already reported, the effects of superatmospheric O_2 and moderate or high CO_2 concentration for fresh products in MAP system are beneficial on the extension of microbial shelf-life of foods (Lee, 2009).

The importance of MAP in muscle foods shelf-life is also useful to improve color, off-flavors, and spoilage (Cooksey, 2009). Many studies have already reported the beneficial effects of MAP (Devlieghere et al., 1999; Grandison et al., 1993; Jacxsens et al., 2001; Laury et al., 2007; Lee et al., 2001; Lee et al., 2008a; Vihavainen et al., 2007; Wang et al., 1983; Yam et al., 2005).

The most commonly encountered definition of intelligent packaging is provided by the European study 'Evaluating Safety, Effectiveness, Economic-environmental Impact and Consumer Acceptance of Active and Intelligent Packaging (ACTIPAK-FAIR CT 98-4170, 1999–2001) as 'systems that monitor the condition of packaged foods to give information about the quality of the packaged food during transport and storage' (Kerry et al., 2006).

Bioactive packaging technologies could be a potential alternative solution to prevent the development and spread of spoilage and pathogenic microorganisms, as well as to extend shelf-life of meat products (Coma, 2008).

Active packaging has been defined as “packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system” (Robertson, 2006a). Indeed, active packaging systems and shelf-life in food research, included muscle foods, is supported by a various papers (Aymerich et al., 2008; Coma, 2008; Kerry et al., 2006).

1.7 Bioflavonoids

Flavonoids, or bioflavonoids, include a large group of polyphenolic compounds that are characterized by a benzo-γ-pyrane, the generic structure of flavonoids and the numbering system used to distinguish the carbon positions around the molecule. The three phenolic rings are A, B, and C (or pyrane) (Cook et al., 1996). Bioflavonoids are universally ubiquitous in food plant (vegetables and fruits) (Yao et al., 2004). Flavonoids cannot be synthesized by humans and animals but are part of human diet. More than 5000 different plant-derived flavonoids have been isolated from various plants. The major flavonoid classes include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones. Flavonoids are absorbed from the gastrointestinal tracts of humans and animals and are excreted either unchanged or as flavonoid metabolites in the urine and feces (Cook et al., 1996). Bioflavonoids are potent antioxidants, free radical scavengers, and metal chelators and inhibit lipid peroxidation. The structural requirements for the antioxidant and free radical scavenging functions of flavonoids include a hydroxyl group in carbon position three, a double bond between carbon positions two and three, a carbonyl group in carbon position four, and polyhydroxylation of the A and B aromatic rings (Cook et al., 1996). Moreover, comprehensive reviews on the healthy

potentiality of these bioactive compounds have demonstrated their cancer preventive effects (Yang et al., 2001).

1.7.1 Use of natural antioxidants and foods

The role of antioxidants in foods is to retard or control oxidation. Natural antioxidants occurring in foods may be used as a ingredient of food formulations, in order to stabilize them or may be extracted and added to foods (Shahidi, 2000). Plants and their biologically active chemical constituents, called secondary metabolites or bioactives, can improve livestock production through dietary supplementation. Therefore, many natural bioactive compounds have antioxidant activities that could improve the oxidative stability of animal food products, like poultry meat and eggs. They may stimulate immunity directly, improving birds' resistance to disease (Wallace et al., 2010). Among several vegetal products, green tea and grape seed extract exhibit the highest level of antioxidant activity, which have been attributed to the higher amount of caffeic acid and epicatechin in green tea and epicatechin and catechin in grape seed extract (Rababah et al., 2004). Furthermore, Teixeira et al. (2013) and Ye et al. (2013) have determined the antioxidant activity, by free radical scavenging activity and ferric reducing power, and the effectiveness of different essential oils (basil oil, carrot seed, celery seed oil, citronella oil, clove oil, coriander oil, garlic oil, grapefruit oil, lemon oil, marjoram oil, onion oil, oregano oil, parsley oil, rosemary oil, sage oil, tarragon oil thyme oil) to inhibit the growth of seven food-borne spoilage and pathogenic bacterial strains (*Brochothrix thermosphacta, Escherichia coli, Listeria innocua, Listeria monocytogenes, Pseudomonas putida, Salmonella typhimurium and Shewanella putrefaciens*). Flavonoids isolated from the rhizomes of *Zingiber spectabile*, have also shown a high antioxidant potential and antibacterial activities (Sivasothy et al., 2013). This would

suggest the remarkable benefit of consuming antioxidant, which is a promising source of natural preservation strategy for the food industry.

1.7.2 Occurrence of bioflavonoids compounds in meat and meat products

The content of bioactive compounds (or bioflavonoids) in meat and in meat products is exclusively related to dietary supplementation and direct addition to food products.

Nowadays, there is a growing interest for using natural extracts from fruits, spices, seeds, grains and herbs in animal breeding. These plant extracts appear as promising substitutes of synthetic compounds and antibiotics widely used as growing factors in the past and recently banned by European Union (Van Horne et al., 2008). It has been widely observed that plant extracts exhibit high antioxidant and antimicrobial activities and, for these reasons, they are used in animal feeding for improving health status of the birds, but also for increasing shelf-life of food products by retarding microbial spoilage and oxidative processes (Friedman et al., 2002; Rababah et al., 2004). Wine processing by-products (grape skins and seeds) are particularly rich in a wide range of polyphenols (proanthocyanidins) and have been used in broiler chicken diets; an addition level lower than 60 g/kg (corresponding to 5.2 g of total polyphenols/kg of feed) did not impair growth performances, while it was able to determine an increase *in vivo* of antioxidant capacity, as well as a lower susceptibility of the meat to undergo lipid oxidation (Brenes et al., 2008; Wang et al., 2008). Grape seed extract also evidenced a good potential as shelf-life extending antioxidant in raw and mainly ground poultry meat, by reducing oxidative processes and retarding lipid oxidation-related volatile undesirable compounds (Rababah et al., 2006; Brannan, 2009). In general, although all of these comprehensive papers are currently available, research

information is still limited and the mechanism underlying the effectiveness of using of plant extracts needs to be more supported. Studies are requested to clarify the relationships among the use of vegetal extracts, the health and welfare of animals under intensive breeding conditions, as well as the relationships between the use of plant extracts and quality characteristics of raw and processed meat, considering the overall lipid oxidation and microbiological shelf-life and the functional properties of different kinds of meat.

1.7.3 Biological effects of bioflavonoids

There has been an increasing interest in the research of flavonoids from dietary sources, due to growing evidence of their versatile health benefits.

Epidemiological studies revealed that Mediterranean diet, abundant in flavonoid-rich foods (*Allium* and *Brassica* vegetables, and red wine), correlated with the increased longevity and decreased incidence of CVD. The most frequently studied flavonoid, quercetin, has demonstrated to have an sparing effect on the cardiovascular system. Quercetin and other flavonoids have been shown to modify eicosanoid biosynthesis (antiprostanoid and anti-inflammatory responses), protect low-density lipoprotein from oxidation (prevent atherosclerotic plaque formation), prevent platelet aggregation (antithrombotic effects), and promote relaxation of cardiovascular smooth muscle (antihypertensive effect). In addition, flavonoids also have antiviral and carcinostatic properties (Formica et al., 1995).

Many flavonoids have antioxidant activity, free radical scavenging capacity, and anticancer activity, while some flavonoids exhibit potential for anti–human immunodeficiency virus functions (Yao et al., 2004). Chinnam et al. (2010) have demonstrated the link of dietary benefits of bioflavonoids on the inhibition of ATP synthase. This research group studied the effects of several

bioflavonoid compounds on *E. coli* ATP synthase inhibition and observed that the extent of inhibition by bioflavonoid compounds was variable. Morin, silymarin, baicalein, silibinin, rimantadin, amantidin, and epicatechin gave a complete inhibition.

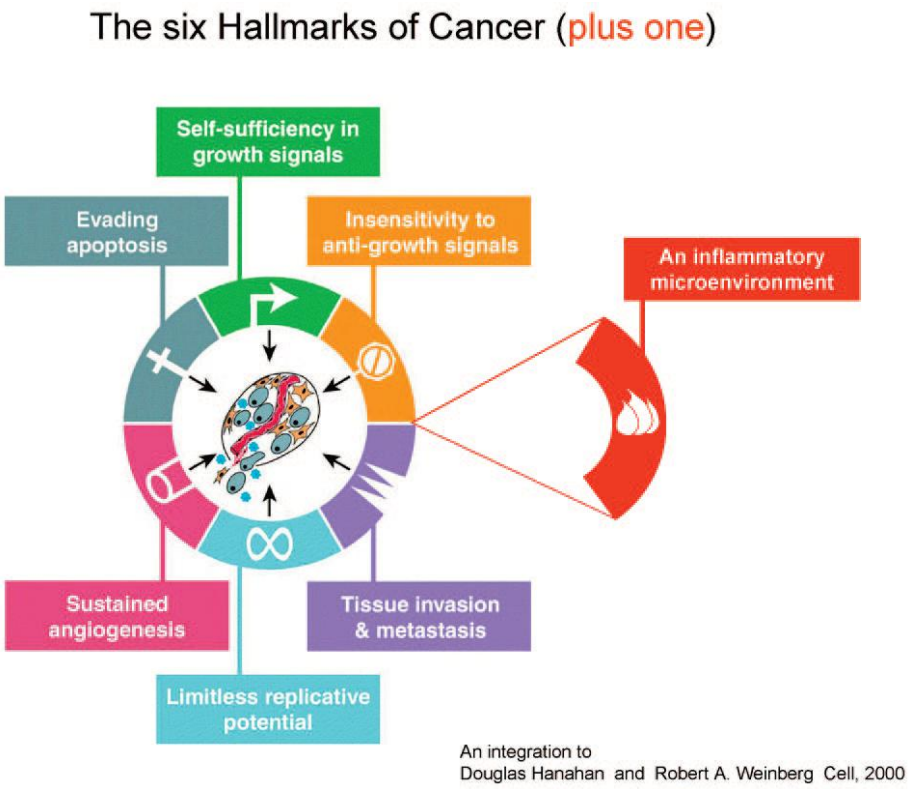
Moreover, Wang et al. (2013) isolated bioflavonoid glycosides from leaves of *Malus hupehensis* (a medicinal herb widely distributed throughout southern China), known as 'Tea crabapple'. Their antioxidant activities, protective effects against doxorubicin-induced cardiomyopathy in H9c2 cells and protective influence against doxorubicin-induced cell death, was strong in these compounds. In a recent *in vivo* study, Lai et al. (2013) demonstrated a wide spectrum of biological activities of an extract of citrus peel. Gold lotion (GL), a formulated product from citrus peel extract, has exhibited remarkable inhibitory effects on azoxymethane-induced colonic tumorigenesis. Both gene and protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were suppressed by GL treatment. These data have revealed that the citrus peel extract GL is an effective antitumor agent that mechanistically downregulates the protein levels of iNOS, COX-2, ornithine decarboxylase, vascular endothelial growth factor, and matrix metallopeptidase 9 in colonic tissues of mice, suggesting that GL is a novel functional natural product capable of preventing inflammation-associated colon tumorigenesis. Moreover, Spanou et al. (2012) have isolated flavonoid glycosides from legume plant extracts, rich in polyphenolic compounds that inhibit xanthine oxidase (XO) activity. XO is the main contributor of free radicals during exercise and in various pathological conditions (hypertension, atherosclerosis, diabetes and cancer).

However, more comprehensive reviews on the anti-inflammatory, antioxidant and cancer preventive effects of bioflavonoids are currently available (Cerella et al. 2010; Hämäläinen et al., 2011; Murakami et al., 2012; Pan et al., 2009; Xiao et al., 2009 and Zhang et al., 2011).

1.7.4 Combinational effects of dietary bioactive components in inhibition of inflammation

Inflammation is associated with cancer and other chronic diseases, therefore inflammatory cells and soluble factors are present in many tumors as the end stage of inflammation in adults (Demaria et al., 2010). The functional link between inflammation and cancer started on 1863 when Virchow hypothesized that the origin of cancer was at sites of chronic inflammation. Furthermore, tumor cells may take over key mechanisms by which inflammation interfaces with cancer, to advance their colonization of the host (Coussens et al., 2002). An inflammatory component is present also in the microenvironment of tumors. Recent studies (Colotta et al., 2009; Coussens et al., 2002; Kim et al., 2009; Mantovani et al., 2008; Mantovani, 2009a) have found a reasonable relationship between inflammation and cancer, suggesting an additional mechanism called cancer-related inflammation (CRI) as the seventh hallmark to the other six identified by Hanahan et al. (2000) (Figure 2, Mantovani, 2009b).

Figure 2: Inflammation as the seventh hallmark of cancer. An integration to the six hallmarks of cancer (from Mantovani (2009b), adapted from Hanaham et al., 2000).



Therefore, in the tumor microenvironment, smoldering inflammation contributes to proliferation and survival of malignant cells, angiogenesis, metastasis, subversion of adaptive immunity, reduced response to hormones and chemotherapeutic agents (Colotta et al., 2009). Many dietary bioactive compounds have been identified as potential anti-inflammatory agents, but their mechanisms of action have not been fully investigated. In this context, it is possible to test anti-inflammatory effects of several dietary bioactive compounds by using *in vitro* techniques.

The use of single bioactive compounds to inhibit inflammation may need an high-dose concentration, causing toxicity for human health. The combination of various agents with different molecular targets may produce synergistic interactions and result in better biological effects (Xiao et al., 2008a). Several studies have proved the potential enhancement of cancer inhibition by using

statins in combination with dietary and pharmacological agents (Xiao, 2009; Xiao et al., 2008b). Therefore, mechanisms of combined action of different chemopreventive dietary compounds may potentially result in more anticancer effects than isolated compounds (De Kok et al., 2008).

Harris et al. (2006) hypothesized that differences in antioxidant activity between the structurally similar flavones, luteolin and chrysin (differing only in B-ring hydroxylation patterns), would diversely affect inflammation-associated Cox-2 expression and prostaglandin E2 (PGE2) formation. Pretreatment of RAW 264.7 macrophage-like cells with different concentrations of luteolin inhibited lipopolysaccharide (LPS)-induced Cox-2 protein expression, while single chrysin pretreatment did not reduce the level of this pro-inflammatory protein. Conversely, both luteolin and chrysin completely suppressed LPS-induced PGE2 formation (Harris et al., 2006). Synergistic anti-inflammatory effects of luteolin and chicoric acid, two abundant constituents of the common dandelion (*Taraxacum officinale*), were investigated in lipopolysaccharide (LPS) stimulated RAW 264.7 cells (Park et al., 2011). Co-treatment with luteolin and chicoric acid synergistically reduced cellular concentrations of nitric oxide (NO) and PGE2 and also inhibited expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). In addition, co-treatment reduced the levels of proinflammatory cytokines, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β (Park et al., 2011).

2 Effects of rearing systems on the quality and oxidative stability of poultry meat

2.1 Abstract

The aim of this research was to evaluate the effect of two different rearing systems (free-range, FR and conventional, C systems) on the oxidative stability and quality properties of chicken breast and thigh meat. FR female and male chickens, belonging to medium growing Isa strain, were raised for 56 and 70 d; C female and male birds were fast growing hybrids (Ross 708) and were separately raised for 39 and 50 d. Female and male chickens were slaughtered into 2 separate sessions, to obtain the main two commercial categories (Rotisserie and Cut-up, respectively). After slaughtering, 12 carcasses of each group were randomly selected and used to evaluate oxidation stability of breast and thigh meat. Lipid content, peroxide value (PV) and thiobarbituric acid reactive substances (TBARs) were determined. In both categories, rearing system did not significantly affect the lipid content of thigh meat (10.4-11.6%), but the C system led to a significantly higher lipid content (0.9-1.7%) in breast meat in both categories. In general, a low level of PV (0.79-1.06 and 0.30-1.29 meq O_2/kg of lipid in breast and legs, respectively) and TBARs (0.06-0.19 and 0.10-0.13 mg MDA/kg of sample in breast and thigh, respectively) were found, regardless of the commercial category. However, the C system significantly increased PV in Rotisserie leg meat, whereas TBARs was significantly higher in Rotisserie breast meat obtained with the FR system. In the Cut-up category, no significant effects on the oxidation stability of thigh meat, were detected, but the FR system led to a significantly higher TBARs content in breast meat. Despite these differences, it can be concluded that the two different rearing systems led to a low oxidation level in poultry meat, which ensures their quality and safety from the consumer health standpoint.

2.1 Introduction

Over the past few years, the concept of food has undergone a radical transformation, as its safety and impact in human health has become more and more important. Poultry breeding systems have been influenced by consumer's priorities, as more attention is being paid to birds raised with organic products, without using of antibiotics or synthetic chemicals. Following the multiple and growing demand of consumers who are more sensitive to the ethical and cultural aspects of foods from animal origin, there is an increasing interest towards unconventional rearing systems and animal-friendly production systems, which can improve animal welfare as well as guarantee higher qualitative standards concerning food safety, nutritional, and sensory properties (Castellini et al., 2008; Magdelaine et al., 2008). EC Regulation 1538/91 have defined processing and marketing standards for poultry meat produced using alternative farming systems (extensive indoor/barn-reared, free-range, traditional free-range and free-range, total freedom), while organic poultry production have been ruled in 1999 (EC Regulation 1804/99). Factors that have fostered this development include the incredible current impact on public opinion of the media on the relationship between diet and health, the growing life-expectancy of the population, with major concern about disease prevention (Jimènez-Colmenero et al., 2001). Understanding consumer perception of risk and impact on purchase behavior is a key issue for the mutual benefit of both consumers and food industry (Yeung et al., 2001). The prospected correlation between high meat intake and human health problems, such as obesity, cardio-vascular and cancer diseases, has led to a reduction of red meat consumption (Schönfeldt et al., 2008). Throughout the years, the poultry industry has changed and adapted to meet the consumer demands of meat products. Regarding nutritional aspects and human health, poultry meat fits well the current consumer request for a

low-fat meat (Barroeta, 2007), but there is a growing demand to improve its nutritional value and the animal wellness with suitable dietary strategies (Bou et al., 2009). Among meat macronutrients, the lipid fraction has the highest susceptibility to modifications. Lipid peroxidation, commonly known as lipid oxidation, is considered one the most important factors affecting the quality of food (Esterbauer, 1993; Kanner, 2007). It is also a major cause of chicken meat quality deterioration, as it can impact shelf life of meat and meat products. The major lipid class in meat adipose tissue (>90%) are triacylglycerols or neutral lipids. In muscle foods, lipid peroxidation has been reported to initiate and propagate primarily in the phospholipid fraction of cell membranes, due to the high content of polyunsaturated fatty acids (Wood et al., 2008). Rhee et al. (1996) observed that raw poultry meat is less prone to lipid oxidation than beef or pork meat.

Since consumers are aware of the importance of animal welfare, there is nowadays an increasing demand for poultry meat and eggs produced from free-ranged animals. The relevance and effects of rearing systems and dietary supplementation on quality broiler meat has been emphasized in several studies (Kralik et al., 2005; Chen et al., 2013; Fanatico et al., 2008; Leusink et al., 2010).

The aim of this study was to evaluate the effect of two different rearing systems (free-range, FR and conventional, C systems) on the oxidative stability and quality properties of chicken breast and thigh meat.

2.2 Materials and Methods

Solvents and reagents

Ammonium thiocyanate (NH_4SCN, $\geq$ 97.5%), barium chloride dehydrate ($BaCl_2 \cdot 2H_2O$, $\geq$ 99%), ethylenediamine-tetraacetic acid (EDTA) disodium salt (100%±1%) were supplied by Curtin Matheson Scientific Inc. Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, $\geq$ 99.0%), trichloroacetic acid (TCA) ($\geq$ 99%), ascorbic acid and double distilled water, were purchased from Carlo Erba Reagenti (Rodano, Italy). 2-Thiobarbituric acid (TBA) minimum 98%, sodium phosphate monobasic anhydrous (NaH_2PO_4), anhydrous sodium sulfate, potassium hydroxide and tridecanoic acid methylester (about 98%) were supplied by Sigma-Aldrich (St. Louis, USA). Sodium phosphate dibasic anhydrous (Na_2HPO_4) was purchased from Fisher Scientific (New Jersey, USA).

Chloroform, *n*-hexane, methanol and potassium chloride ($\geq$ 99%) were supplied by Merck (Darmstadt, Germany). Anhydrous sodium sulfate and potassium hydroxide were purchased from BDH (Poole, England) and Prolabo (Fontenay, France), respectively. The standard mixture of fatty acid methyl esters (GLC 463) was supplied by Nu-Chek (Elysian, MN, USA).

The phosphate buffer used for the TBARs determination, was prepared by adding 65.8 mL of 0.5 M NaH_2PO_4 and 111 mL of 0.5 M $Na_2HPO_4 \cdot H_2O$ (water solutions) in a 500 mL volumetric flask. pH was controlled, taken to neutrality (either with 1 N HCl or 1 N NaOH solutions), and made up to volume with water. To delay oxidation and prevent the pro-oxidative effect of metals, proper amounts of EDTA and ascorbic acid were added to the phosphate buffer to reach a final concentration of 0.1% (w/v) of both of them.

To prepare the iron(II) chloride solution used for PV determination, 0.4 g barium chloride dihydrate were dissolved in 50 mL of water. This solution was slowly added and with constant stirring to an iron(II) sulfate solution (0.5

g FeS04·7H20 dissolved in 50 mL of water). Two milliliters of 10 N HCl solution was added to the resulting solution. The barium sulfate precipitate was filtered to give a clear iron(II) solution, which was stored in a brown bottle and kept in the dark. To prepare the ammonium thiocyanate solution, 30 g of ammonium thiocyanate was dissolved in water, and the volume was made up to 100 mL.

Experimental design

The study was carried out on 96 chickens, which are divided into two experimental groups. The first group (n=48) was composed by Ross 708 chickens (fast-growing genotype); which were reared indoor in a conventional (C) way, under intensive conditions; the birds were kept in a poultry house under controlled environmental conditions and without outdoor access. C female and male birds were separately raised for 39 and 50 d, respectively. Chicks were fed the same diet (0 to 14 d: starter; 15 to 35 d: grower; 36 to 50 d: finisher), as described in Table 4.

The 2nd group (n= 48) was composed by Hubbard Red (Isa) chickens (slow-growing genotype), which were kept outdoor under a free range (FR) system; FR female and male chickens were separately raised for 56 and 70 d, respectively.

Female and male chickens were slaughtered into 2 separate sessions, in order to obtain the main two commercial categories (rotisserie and cut-up, respectively). After slaughtering, 12 carcasses of each group were randomly selected and used to evaluate oxidation stability of breast (skinless) and leg meat (with skin). Each sample was removed from the carcass, minced, packed in plastic bags covered by aluminum foil under vacuum and kept at –18°C until analysis.

The experimental design is reported in Table 5.

Table 4: Main ingredients and composition of experimental diets.

	Conventional diet			Free range diet			
	Starter	Grower	Finisher	Starter	Grower	Finisher	Finisher
	0-14 d	15-35 d	36- 50 d	0-14 d	15-35 d	36-54 d	54-70 d
Main ingredients (%)							
Corn	20.0	5.0	5.0	20.0	5.0	5.0	50.0
Wheat	35.8	57.0	59.9	39.5	60.9	65.8	
Soybean meal	29.7	24.1	21.8	22.6	20.3	16.6	
Sunflower meal				2.2			
Wheat bran	3.0	3.0	3.0	10.0	3.0	3.0	
Sulflower oil	5.0	7.1	7.0				
Soybean					3.0	3.0	
Soybean oil				1.5	3.7	3.3	
M2661							50.0
Calculated composition							
Energy (MJ of ME [a]/kg)	12.8	13.3	13.4	11.9	12.6	12.7	13.0
Dry matter (%)	88.2	88.4	88.3	87.7	88.1	88.1	87.8
Crude protein (%)	22.1	19.3	18.5	19.6	18.9	17.5	16.5
Lipid (%)	6.8	8.6	8.4	3.5	5.7	5.4	5.3
Crude fiber (%)	2.5	2.5	2.5	3.0	2.6	2.5	2.4
Ash (%)	5.5	4.8	4.4	5.6	5.1	4.4	4.5

[a] ME: Metabolizable Energy.

Table 5: Experimental design.

	Conventional		Free range	
Trait	Thigh	Breast	Thigh	Breast
Genotype	Ross 708		Isa	
Sex	Female	Male	Female	male
Stocking density (birds/sqm)	18	10	13	7
Outdoor access	-	-	From 28 to 56 d	From 28 to 70 d
Age at slaughter	39	50	56	70
Slaughter weight	1.9 kg	3.1 kg	1.8 kg	2.8 kg
Carcass yield	65 %	71 %	62 %	68 %
Carcass weight	1.2-1.3 kg	2.2-2.3 kg	1.1-1.2 kg	1.9-2.0 kg

Lipid extraction

Lipids were extracted according to a modified version (Boselli et al., 2001) of the method described by Folch et al. (1957). The frozen samples were minced and were homogenized (6575 g/rfc or 21500 rpm, for 3 min) 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 thermostated at 60°C for 20 min before adding 100 mL chloroform. After 2 min of homogenization (6575 g/rfc or 21500 rpm), the content of the bottle was filtered through filter paper to eliminate the solid residue, which consisted mostly of proteins. The filtrate was mixed thoroughly with 100 mL of a 1 M KCl solution and left overnight at 4°C in order to obtain phase separation. The lower phase containing the lipids was collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Two lipid extractions were performed for each sample.

GC-FID determination of total fatty acid methyl esters (FAME)

About 20 mg of lipid extract were methylated with 200 μL of diazomethane (Fieser and Fieser, 1967); 1.01 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, left standing 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) interfaced with a computerized system for data acquisition (Chromcard Data System, ver. 2.3.1, Fisons Instruments). A RTX 2330 fused-silica column (105 m x 0.25 mm x 0.2 μm film thickness) (Restek, Bellefonte, PA) coated with 90% biscyanopropyl- and 10% cyanopropylphenyl-polysiloxane was used. Oven temperature was programmed from 100°C to 240 C, 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. Two replicates were run per sample.

Tridecanoic acid methyl ester was used as internal standard for FA quantification, and 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 (C13:0). The limit of detection (LOD) of FAMEs was 0.0035 mg, whereas the limit of quantification (LOQ) was 0.011 mg. LOD and LOQ were calculated as a signal-to-noise ratios equal to 3:1 and 10:1, respectively.

Spectrophotometric determination of peroxide value

PV was determined using a modified method of Shantha and Decker (1994). Briefly, 20 mg of extracted lipids were mixed with 9.8 mL of

choloform:methanol (2:1, v/v) and 50 μL of thiocyanate/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 0.1-5 μg/mL (y = 0.0311x - 0.0375; r^2 = 0.998). Peroxide value was expressed as meq O_2/kg fat. Two replicates were run per sample.

Spectrophotometric determination of thiobarbituric reactive substances (TBARs)

TBARs were used as an indicator of the secondary lipid oxidation products using the method of Tarladgis et al. (1960). 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) trichloroacetic acid aqueous solution were then added and sample was homogenized followed by filtration. Five mL of 0.02 M aqueous solution of thiobarbituric acid were added to 5 mL of the resulted solution, in the capped tubes stored at 90°C for 20 min, followed by refrigeration. 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 0.03-2.26 μg mL^{-1} (y = 0.0015x - 0.0078; r^2 = 0.999). TBARs value was expressed as mg of MDA kg^{-1} of sample. Two replicates were run per sample.

Statistical analysis

Data were analyzed by means of one-way-analysis of variance ANOVA (GLM/PASW procedure) to test the effect of production system (conventional vs. free-range) on meat quality traits within each carcass type (rotisserie and cut-up). Overall differences between rearing means were tested according to Tukey's test, performed at 95% confidence level and considered to be significant when $P < 0.05$ (PASW Statistics, 17).

2.3 Results and Discussion

Lipid content

Table 6 shows the effects of rearing systems on the average lipid content of the breast (skinless) and thigh (with skin) meat. The statistical analysis shows that in the rotisserie and cut-up categories, the conventional rearing system led to a significantly higher lipid content in breast meat (0.9-1.7%). In thigh meat, on the contrary, the rearing system did not significantly affect the lipid content (10.4-11.6%).

Wang et al. (2009) found that nutrient composition (water, protein, and fat) of the chicken muscle were not influenced ($P > 0.05$) by the FR system, but the latter had a significant impact on reduction of abdominal fat. Moreover, Fanatico et al. (2005) demonstrated that *pectoralis* dry matter (%), fat (%) and ash (%) were not affected ($P > 0.05$) by genotype or outdoor access, demonstrating that free range production system had a limited effect on fat content of breast meat. Castellini et al. (2002), in agreement with Wang et al. (2009), showed that FR rearing could favor a higher energy consumption, improving lipogenesis and increasing motor activity that leads to a less abdominal fat on carcass traits. The latter can be explained considering that neutral lipids (triacylglycerol, TAG), mainly rich in saturated and monounsaturated fatty acids, are found in the intramuscular adipocytes (adipose tissue) located in the perimysium (Sanosaka et al., 2008). Breast

meat contains more phospholipids than thigh meat, where the predominant lipid class are TAG (Gonzalez-Esquerra et al, 2001). However, a large data variation on intramuscular fat content of breast muscles has been observed in literature, which might be attributed to both sampling and analytical procedures used (Cortinas et al., 2004).

Lipid content of breast meat found in the present study are in agreement with those of previous works (Barroeta, 2007; Du et al., 2002). Moreover, our results are consistent with recent studies that show how outdoor rearing can reduce muscle fat content (Chen et al., 2013; Fanatico et al., 2007; Bogosavljevic-Boskovic et al., 2010).

Table 6: Lipid content of breast and thigh meat of Rotisserie and Cut-up carcasses from conventional and free range production systems (mean ± ds). Each value is the average of twelve independent replicates.

Trait	Rotisserie carcass		Cut-up carcass	
	Conventional	Free range	Conventional	Free range
Breast (n.) skinless	12	12	12	12
Lipid content (%)	1.04 ± 0.10^{a}	0.89 ± 0.09^{b}	1.71 ± 0.12^{a}	1.07 ± 0.12^{b}
Thigh (n.) with skin	12	12	12	12
Lipid content (%)	11.56 ± 1.22^{a}	10.80 ± 1.11^{a}	10.40 ± 1.47^{a}	10.73 ± 1.23^{a}

$^{a-b}$ = *P<0.05*

Fatty acid composition

Tables 7 and 8 report the effects of rearing systems on the fatty acid composition (expressed as %) of breast and thigh meat chicken. In rotisserie and cut-up breast meat, the most abundant FA was oleic acid (~26-28% and ~27-32% of total FA), followed by linoleic (~23-25% and ~26-29%), palmitic (~20-23% and ~19-24%), and stearic acids (~9% and ~7-9%). Rotisserie and cut-up thigh meat showed a similar FA abundance order with respect to breast meat, but with different internal percentages; in fact, the main FA was also oleic acid (~35-37% and ~34-35% of total FA), followed by linoleic (~26-30% and ~28-30%), palmitic (~18-21% and ~19-21%), and stearic acids (~6% and ~5-6%) in rotisserie and cut-up thigh meat, respectively. Among long-chain polyunsaturated FA (PUFA), arachidonic acid was the most abundant FA in rotisserie and cut-up breast meat (~5-7% and ~2-5% of total FA) followed by linolenic (~1-2% and ~2-3%) and docosahexaenoic (DHA) acids (< ~1%, in both categories), while no eicosapentaenoic acid was detected. On the other hand, thigh meat displayed different PUFA composition with regard to breast, as the main long-chain PUFA was linolenic acid (~2-3% of total FA), followed by arachidonic (< ~1%) and docosahexaenoic (DHA) acids (~0.06%) in both rotisserie and cut-up categories. Furthermore, in breast meat, PUFA was the most abundant FA class (~37-38% in both categories), while saturated FA (SFA) accounted for ~30-33% and ~27-33%, and monounsaturated FA (MUFA) for ~30-33% and ~30-36% of total FA, in rotisserie and cut-up, respectively. On the contrary, the most relevant FA class in thigh meat were MUFA (~40-42% and ~39-40% of total FA, in rotisserie and cut-up, respectively), followed by PUFA (~30-34%) and SFA (~25-30%).

As shown in Tables 7 and 8, the FA profile of free-range rearing system was significantly more saturated than the conventional one (except for cut-up thigh meat), which can be mainly ascribed to the palmitic acid content. On the

other hand, the higher MUFA level of breast and thigh meat obtained by conventional rearing, was mainly due to the oleic acid content. C and FR systems differently affected breast and thigh fatty acid composition in rotisserie and cut-up categories, despite the data variability due to the nature of samples.

The statistical analysis of the single FA content (Table 8) demonstrated that palmitic, arachidonic and DHA acids were significantly higher in FR rearing with respect to C in both categories of breast meat. The C system led to a significantly higher amount of linoleic and linolenic acid in breast meat rotisserie category, while the cut-up one showed a significantly higher level of palmitoleic, stearic, oleic, linoleic and linolenic acid. FR system resulted in a significantly higher amount of palmitic, arachidonic and DHA acids in breast meat cut-up category.

Rearing had significant effects in the FA composition of thigh meat (rotisserie category), as the amount of palmitic, palmitoleic, stearic, oleic and arachidonic acids were higher in FR system, while the linoleic and linolenic acids were significantly greater in samples from C one. On the other hand, thigh meat from the cut up category showed a significantly higher level of myristic and arachidonic acids in FR system and a higher concentration of linolenic acid in the conventional one.

Cortinas et al. (2004) also observed a higher incorporation of PUFA in breast than in thigh meat. These differences could be ascribed to the role of FA in these tissues or to the phospholipids contents (Hulan et al., 1988; Ratnayake et al., 1989)

Betti et al. (2009a) evaluated the FA distribution between TAG and PL classes in n-3-enriched broiler meat, finding that linolenic acid was mainly deposited in the TAG fraction of both breast and thigh meat skinless. Betti et al. (2009a) reported similar results to those obtained in the present study, where the main PUFA n-3 is linolenic acid. Gonzalez-Esquerra et al. (2001)

stated that linolenic acid is not stored in PL membranes of chicken breast muscle.

The statistical analysis of the FA classes demonstrated that SFA, n-6/n-3, SFA/PUFA and Δ-desaturase index were significantly higher in FR rearing with respect to C in both categories of breast meat. Inversely, MUFA and UFA/SFA ratio were significantly higher in both categories of breast meat produced with the conventional system.

In thigh meat of rotisserie category, SFA, MUFA, n-6/n-3, SFA/PUFA and Δ-desaturase index were significantly higher in FR system and only UFA/SFA rate was significantly higher in the conventional one. The thigh cut-up category, had a significantly higher n-6/n-3, SFA/PUFA and Δ-desaturase index when obtained by FR rearing, but there was a higher concentration of PUFA n-3 in thigh meat produced with C system. PUFA n-6/n-3 ratio ranged from 8.7 to 10.2 (breast) and 9.7 to 12.7 (thigh), which are clearly higher than the suggested ratio (n-6:n-3 ratio < 4) for a healthy human diet (Simopoulos, 1999). The balance of omega-6/omega-3 fatty acids is an important determinant in decreasing the risk for coronary heart disease, both in the primary and secondary prevention of coronary heart disease (Simopoulus, 2008).

Δ-desaturase index ((C20:2 n-6 + C20:4 n-6 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3/C18:2 n-6 + C18:3 n-3 + C20:2 n-6 + C20:4 n-6 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3)*100) is useful to evaluate the activity of both Δ^5 and Δ^6-desaturases, which are enzymes that catalyze the formation of PUFA n-6 and n-3. The Δ-desaturase index varied from 22.5% to 28.9% and from 11.4% to 23.0% in the rotisserie and cut-up categories, respectively, exhibiting a higher enzymatic activity in breast meat. Regarding the Δ-desaturase index in both commercial categories of thigh meat, it ranged from 3.6% to 4.5% and from 3.6% to 4.7% in the rotisserie and cut-up categories, respectively. Our data indicate that Δ-desaturase activity levels in thigh meat seems to be 3-6

lower than in breast meat. The reduced Δ^{5-}desaturase activity indicates that linolenic acid suppress bioconversion of arachidonic acid from linoleic acid (Garg et al., 1988). Betti et al. (2009a) demonstrated a strong decrease of Δ^{5-} desaturase activity in poultry meat supplemented by flaxseed.

FA composition of meat from conventional rearing was not similar to those of free-range one, which could be ascribed to several factors. Although the ingredients of the free-range and conventional diets were the same, it must be pointed out that chicken grown with the free-range rearing had access to grassland areas, so they could have eaten grass, insects and worms. This aspect might partly explain some differences between the higher % of PUFA (like arachidonic acid) and more Δ-desaturase activity in meat obtained with FR system, especially in breast. In addition, essential FA (linoleic and linolenic acids) are precursors of the long-chain PUFA n-6 and n-3 series so, besides being absorbed, essential FA are elongated and desaturated to give rise to long-chain PUFA n-6 and n-3 (such as arachidonic and DHA).

Cortinas et al. (2004) have reported a significant effect of dietary polyunsaturation level on the FA composition of poultry meat, proving that such approach produces a decrease of MUFA and SFA content in poultry meat.

Different strategies have been studied to improve oxidative stability and sensory characteristics of meat products through natural dietary supplements (Bou et al., 2009; Betti et al., 2009b) and by ensuring animal welfare (Chen et al., 2013; Fanatico et al., 2008).

Table 7: Fatty acid classes (as % total FA) and their ratios present in breast and thigh meat of Rotisserie and Cut-up categories, from conventional and free range production systems (mean ± ds). Each value is the average of twelve independent replicates.

Trait	Rotisserie carcass		Cut-up carcass	
	Conventional	Free range	Conventional	Free range
Breast (n.) skinless	12	12	12	12
SFA	30.57 ± 2.07[a]	33.34 ± 1.07[b]	27.06 ± 2.18[a]	33.16 ± 3.42[b]
MUFA	32.50 ± 2.94[a]	29.45 ± 2.92[b]	36.24 ± 2.42[a]	30.49 ± 3.13[b]
PUFA	36.89 ± 2.02[a]	37.17 ± 2.32[a]	36.51 ± 1.40[a]	37.88 ± 4.26[a]
n-3	3.67 ± 0.41[a]	3.32 ± 0.43[a]	3.75 ± 0.17[a]	3.54 ± 0.44[a]
n-6	32.87 ± 1.75[a]	33.61 ± 1.97[a]	32.62 ± 1.23[a]	34.14 ± 3.89[a]
n-6/n-3	9.06 ± 0.99[a]	10.24 ± 0.99[b]	8.70 ± 0.17[a]	9.67 ± 0.71[b]
SFA/PUFA	0.83 ± 0.08[a]	0.90 ± 0.05[b]	0.74 ± 0.07[a]	0.88 ± 0.06[b]
Δ-desaturase index	22.51 ± 2.43[a]	28.88 ± 3.51[b]	11.35 ± 2.69[a]	23.00 ± 6.55[b]
Thigh (n.) with skin	12	12	12	12
SFA	25.07 ± 3.62[a]	30.20 ± 8.73[b]	25.86 ± 2.75[a]	27.65 ± 1.67[a]
MUFA	40.45 ± 1.81[a]	41.98 ± 3.24[b]	39.47 ± 2.25[a]	39.62 ± 1.71[a]
PUFA	34.28 ± 3.30[a]	30.16 ± 1.66[a]	34.56 ± 2.30[a]	32.63 ± 2.28[a]
n-3	3.11 ± 0.30[a]	2.21 ± 0.15[a]	3.22 ± 0.22[a]	2.56 ± 0.19[b]
n-6	31.11 ± 3.00[a]	27.89 ± 1.57[a]	31.29 ± 2.09[a]	30.02 ± 2.09[a]
n-6/n-3	10.01 ± 0.30[a]	12.64 ± 0.71[b]	9.73 ± 0.15[a]	11.71 ± 0.25[b]
SFA/PUFA	0.75 ± 0.17[a]	1.00 ± 0.25[b]	0.75 ± 3.65[a]	0.85 ± 0.10[b]

Δ-desaturase index	3.59 ± 0.42^{a}	4.47 ± 0.51^{b}	3.65 ± 0.44^{a}	4.72 ± 0.49^{b}

Abbreviations: C, conventional, FR, free range rearing system; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; Stat. Sign., statistical significance; UFA, unsaturated fatty acids. ns, not significant; *, significant at $P \leq 0.05$; a, b statistically-different means (one way Anova; $P \leq 0.05$)

Table 8: Fatty acid composition (as % total FA) of breast and thigh meat of Rotisserie and Cut-up categories, from conventional and free range production systems (mean ± ds). Each value is the average of twelve independent replicates.

Factors	Fatty acid composition of breast meat (%)																	
	C14:0	C16:0	C16:1 n-7	C18:0	C18:1 n-9	C18:2 n-6	C20:0	C18:3 n-3	C20:4 n-6	C22:6 n-3	SFA	MUFA	Total PUFA	PUFA n-3	PUFA n-6	(n-6)/ (n-3)	UFA/ SFA	SFA/ PUFA	Δ desaturase
Rotisserie																			
C	0.36[a]	20.69[a]	2.58[a]	9.21[a]	27.38[a]	25.40[a]	0.08[a]	1.90[a]	4.63[a]	0.40[a]	30.57[a]	32.50[a]	36.89[a]	3.67[a]	32.87[a]	9.06[a]	2.29[a]	0.83[a]	22.51[a]
FR	0.35[a]	23.07[b]	2.38[a]	9.66[a]	26.43[a]	23.20[b]	0.06[b]	1.09[b]	6.72[b]	0.70[b]	33.34[b]	29.45[b]	37.17[a]	3.32[a]	33.61[a]	10.24[b]	2.00[b]	0.90[b]	28.88[b]
Stat. Sign.	ns	*	ns	ns	ns	*	*	*	*	*	*	*	ns	ns	ns	*	*	*	*
Cut up																			
C	0.38[a]	19.84[a]	3.72[a]	6.58[a]	31.69[a]	28.47[a]	0.07[a]	2.68[a]	2.33[a]	0.25[a]	27.06[a]	36.24[a]	36.51[a]	3.75[a]	32.62[a]	8.70[a]	2.71[a]	0.74[a]	11.35[a]
FR	0.40[a]	23.50[b]	2.97[b]	8.96[b]	26.91[b]	25.81[b]	0.07[a]	1.55[b]	5.51[b]	0.64[b]	33.16[b]	30.49[b]	37.88[a]	3.54[a]	34.14[a]	9.67[b]	2.07[b]	0.88[b]	23.00[b]
Stat. Sign.	ns	*	*	*	*	*	ns	*	*	*	*	*	ns	ns	ns	*	*	*	*
Factors	**Fatty acid composition of thigh meat (%)**																		
	C14:0	C16:0	C16:1 n-7	C18:0	C18:1 n-9	C18:2 n-6	C20:0	C18:3 n-3	C20:4 n-6	C22:6 n-3	SFA	MUFA	Total PUFA	PUFA n-3	PUFA n-6	(n-6)/ (n-3)	UFA/ SFA	SFA/ PUFA	Δ desaturase
Rotisserie																			
C	0.41[a]	18.71[a]	3.91[a]	5.71[a]	35.55[a]	29.60[a]	0.08[a]	2.87[a]	0.70[a]	0.06[a]	25.07[a]	40.45[a]	34.28[a]	3.11[a]	31.11[a]	10.01[a]	3.07[a]	0.75[a]	3.59[a]
FR	0.46[a]	21.02[b]	4.45[b]	6.00[b]	36.75[b]	26.24[b]	0.08[a]	1.99[b]	0.85[b]	0.06[b]	30.20[b]	41.98[b]	30.16[a]	2.21[a]	27.89[a]	12.64[b]	2.52[b]	1.00[b]	4.47[b]
Stat. Sign.	ns	*	*	*	*	*	ns	*	*	*	*	*	ns	ns	ns	*	*	*	*
Cut up																			
C	0.40[a]	19.64[a]	4.27[a]	5.50[a]	34.65[a]	29.77[a]	0.08[a]	2.98[a]	0.75[a]	0.05[a]	25.86[a]	39.47[a]	34.56[a]	3.22[a]	31.29[a]	9.73[a]	2.90[a]	0.75[a]	3.65[a]
FR	0.48[b]	20.99[a]	4.52[a]	5.83[a]	34.50[a]	28.15[a]	0.08[a]	2.30[b]	0.99[b]	0.08[b]	27.65[a]	39.62[a]	32.63[a]	2.56[b]	30.02[a]	11.71[b]	2.63[a]	0.85[b]	4.72[b]
Stat. Sign.	*	ns	ns	ns	ns	ns	ns	*	*	*	ns	ns	ns	*	ns	*	ns	*	*

Abbreviations: C, conventional, FR, free range rearing system; Stat. Sign., statistical significance; ns, not significant; *, significant at $P \leq 0.05$; a, b statistically-different means (one way Anova; $P \leq 0.05$)

Lipid oxidation

Table 9 shows the effects of rearing on the PV and TBARs of breast and thigh meat. In general, a low lipid oxidation level was observed in all samples, which was confirmed by the both primary and secondary oxidation products parameters; these data are in agreement with those reported in literature (Betti et al., 2009a; Barroeta, 2007). In fact, PV (0.79-1.06 and 0.30-1.29 meq O_2/kg of lipid in breast and thigh, respectively) and TBARs (0.06-0.19 and 0.10-0.13 mg MDA/kg of sample in breast and thigh, respectively) were found, regardless of the commercial category. Both oxidation parameters are far below the PV level (20 meq O_2/kg lipids) associated with oil rancidity, and below TBARs level (1 mg MDA/kg of sample) associated with lamb meat rancidity (Ripoll et al., 2011).

However, in the Rotisserie and Cut-up categories, only the free range system led to a significantly higher TBARs content in breast meat. On the contrary, the conventional system significantly increased PV only in Rotisserie thigh meat. In the Cut-up category, the factorial analysis shows no significant differences due to rearing on the oxidative stability of thigh meat. Although it is usually expected that conventional system causes more stress than FR one, a good and similar oxidative stability was observed in samples from both types of breeding systems. Several studies report that dietary polyunsaturation level and processing of meat significantly affect TBARs values (Cortinas et al., 2005).

Castellini et al. (2002) stated that the meat of organic chickens has a considerable amount of malondialdehyde, even though the more natural rearing conditions increase motor activity, favor the development of the muscle mass, reduce fatness, make animals calmer and less sensitive to stressors. The oxidative status of organic animals might be affected by the

intense motor activity as it increases muscle oxidative metabolism and free radical production. Castellini et al. (2006) found a higher TBARs level in Ross 205 chickens with respect to Kabir ones, since the Ross chickens were in general less active and they spent more time indoors than outdoors.

Table 9: PV and TBARs of breast and thigh meat of rotisserie and cut-up carcasses from conventional and free range production systems (mean ± ds). Each value is the average of twelve independent replicates.

Trait	Rotisserie carcass		Cut-up carcass	
	Conventional	Free range	Conventional	Free range
Breast (n.) <u>skinless</u>	12	12	12	12
PV (meqO_2/kg lipids)	1.06 ± 0.36[a]	1.02 ± 0.32[a]	0.79 ± 0.30[a]	0.97 ± 0.32[a]
TBARs (mgMDA/Kg meat)	0.15 ± 0.04[a]	0.19 ± 0.05[b]	0.06 ± 0.02[a]	0.15 ± 0.09[b]
Thigh (n.) <u>with skin</u>	12	12	12	12
PV (meqO_2/kg lipids)	0.46 ± 0.18[a]	0.30 ± 0.11[b]	1.29 ± 1.48[a]	0.66 ± 0.54[a]
TBARs (mgMDA/Kg meat)	0.12 ± 0.03[a]	0.12 ± 0.04[a]	0.10 ± 0.02[a]	0.13 ± 0.06[a]

[a-b] = *P<0.05*

Correlations between fatty acid composition and oxidation parameters of chicken meat

A correlation study (Spearman test, α= 0.05) was performed on the results obtained for fatty acid composition and the oxidation parameters of poultry meat obtained with the two types of rearing systems. For a better data comprehension, only significant correlations are here discussed.

In conventional cut-up breast meat, arachidonic acid contents (r= 0.685, P= 0.05*) and Δ-desaturase index (r= 0.601, P= 0.05*) were linearly correlated to PV level. All these correlations confirm the importance of the FA composition of the dietary fat and FA accumulation on the primary oxidation level of breast meat, since FA unsaturation is known to favor such degradation process, especially in conventional rearing system where rearing conditions might contribute to a higher stress level of animals. These trends evince the importance of increasing antioxidant level by dietary supplementation to improve the oxidative stability of products from animal origin.

In FR cut-up breast, myristic acid (r= -0.618, P= 0.05*) and arachidic acid (r= -0.608, P= 0.05*) were negatively correlated to the PV level, which confirms how oxidation can be limited by the presence of SFA.

In rotisserie thigh meat obtained with both rearing systems, oxidation parameters (PV and TBARs) were influenced by FA composition. In C thigh meat, a negative, linear correlation was found between n-6/n-3 ratio (r= -0.755, P= 0.01**) and PV. This could be related to the relative percentages of PUFA n-6 and n-3, as the major PUFA n-3 is linolenic acid, which is more prone to oxidation than linoleic acid (the main PUFA n-6) due to its higher unsaturation degree. In fact, linolenic acid oxidizes about two times faster than linoleic acid (Gunstone et al., 1986).

Some correlations were also observed in FR cut-up thigh between SFA/PUFA ratio (r= -0.691, P= 0.05*) and PV level, as well as between n-6/n-3 ratio (r= 0.839, P= 0.01**) and TBARs content. All these correlations confirm the important role of SFA/PUFA ratio on limiting the extent of the primary lipid oxidation and the critical role of n-6/n-3 ratio on the evolution of secondary oxidation process and generation of rancidity FR thigh meat.

Moreover, a positive, linear correlation was only found between PV and TBARs in cut-up thigh produced by FR system (r= 0.853, P= 0.01**), which supports the well-known interdependence between hydroperoxides and their demolition/evolution compounds; the extent of such conversion is affected by the balance between prooxidative/antioxidative factors present in the muscle.

Conclusions

In general, a low level of both primary and secondary oxidation products was found in chicken breast and thigh meat samples of two commercial categories (rotisserie and cut-up) obtained with different rearing systems (free-range and conventional). However, the C system significantly increased PV in Rotisserie thigh meat, whereas TBARs was significantly higher in Rotisserie breast meat obtained with the FR system. In the Cut-up category, no significant effects on the oxidation stability of thigh meat were detected, but the FR system led to a significantly higher TBARs content in breast meat.

Despite these differences, it can be concluded that the two different rearing systems led to a low oxidation level in poultry meat, which ensures the oxidative quality of these food products and their quality and safety from the consumer health standpoint.

3 Effects of modified atmosphere packaging and rearing systems on poultry meat shelf-life

3.1 Abstract

The aim of this study was to evaluate the effects of rearing systems (free-range, FR and conventional, C) and packaging conditions on the oxidative stability of poultry thigh meat stored at 4°C for 14 days. FR female and male chickens, belonging to medium growing Isa strain, were raised for 56 and 70 d; C female and male birds were fast growing hybrids (Ross 708) and were separately raised for 39 and 50 d. Female and male chickens were slaughtered into 2 separate sessions, to obtain the main two commercial categories (Rotisserie and Cut-up, respectively). After slaughtering, carcasses of each group were randomly selected and thighs were placed in polystyrene foam trays and either over-wrapped in polyethylene film (ordinary atmosphere, OA) or placed in a pouch made of polyethylene and polyamide injected with a modified gas mixture (72% O_2 28% CO_2, modified atmosphere MA). Four packed samples of each atmosphere type (time zero samples) were immediately removed and kept at –18°C until analysis. The other packed samples were stored at 2-4°C for 14 days in the dark. Two packs of each atmosphere, rearing system and commercial category were sampled every 3-4 days of storage (0, 3, 7, 10 and 14 d) and kept at –18°C for subsequent analysis. In both commercial categories, the rearing systems and packaging conditions did not significantly affect the lipid content of thigh meat (5.5-10.7%), and PV level (0.47-11.46 meq O_2/kg of lipids) during storage. However, TBARs content (0.08-1.24 mg MDA/kg of sample) showed a significant increase in rotisserie FR samples stored under ordinary atmosphere packaging. Moreover, TBARs was significantly higher in both categories of

thigh samples stored for 10 and 14 days. The two different rearing systems led to a low secondary oxidation level (0.18-0.42 and 0.16-0.19 mg MDA/kg of sample in rotisserie and cut-up respectively) in samples stored up to 7 days with both packaging conditions. Overall, half shelf-life of all thigh samples ensures their quality and safety from the consumer health standpoint.

3.1 Introduction

Poultry meat quality is a very important issue, as it involves physico-chemical, microbial and sensory characteristics that should match the consumer's quality expectations (Van Boekel, 2008). Rearing systems have been modified and improved so as to meet market demand for high-quality products and legal poultry welfare requirements (Bogosavljevic-Bokovic et al., 2012). One of the main factors affecting chicken meat quality is lipid oxidation, which not only influences sensory and nutritional quality of foods, but also impacts the food product's shelf-life (Yanishlieva et al., 1999). Lipid oxidation starts immediately during breeding and after slaughtering, whose magnitude, will greatly depend on the amount of free radicals present in the muscle as well as on its total antioxidant capacity (Castellini et al., 2006). Such characteristics can be modulated through appropriate breeding and dietary supplementation strategies. Another aspect that helps keeping meat quality is packaging, which makes food more convenient and gives it greater safety assurance from microbial and physico-chemical changes during storage; this will have a direct effect on meat shelf-life and it may extend it. As a result, packaging has become an indispensable element in meat manufacturing process (Skandamis et al., 2002). Consumers indeed demand high quality, natural, nutritious, fresh appearance and convenient meat products with natural flavor, taste and a prolonged shelf-life (Aymerich et al., 2008). The choice of packaging conditions (atmosphere and film) is therefore

a crucial issue. Modified atmosphere packaging (MAP) and vacuum are both used for meat and meat products.

Carbon dioxide is used in MAP to extend the shelf-life of perishable foods, because it inhibits pathogenic microbial growth (gram-negative bacteria and molds) especially under low temperature (Lee et al., 2008b). In a similar way, dissolved O_2 affects the microbial growth of anaerobic microorganisms, but it maintains a desirable color of meat. However, high levels of oxygen alone present in food packages may facilitate microbial growth (especially aerobic microorganisms) and, although oxygen sensitive foods (like meat) can be packaged under MAP or vacuum conditions, such techniques do not always facilitate the complete removal of oxygen (Kerry et al., 2006). Residual oxygen can favor various degradation phenomena, such as lipid oxidation and sensory changes (mainly flavor and color), and thus impact meat shelf-life. While red meats would rapidly discolor due to metmyoglobin formation, the colors imparted to poultry meat by metmyoglobin and methemoglobin are usual and acceptable for fresh poultry meat (Pettersen et al., 2004). Meat is usually placed in small packages with a transparent plastic film/cover to show the food product are more and more appreciated; however, this favors meat photo-oxidation as reported by different studies (Coma, 2008; Boselli et al., 2012; Boselli et al., 2005). The thickness, gas (O_2) and water permeability of the plastic film, in combination with the storage temperature, will greatly impact the oxidative stability of meat (Lee, 2009; Lee et al., 2008b), as it will affect the gas concentrations inside the package, and their absorption or dissolution into foods. Therefore, overall shelf-life extension conferred by proper packaging is one of the most important quality criterion, especially for fresh meat products. Keokamnerd et al. (2008) determined the combined role of herb spices and packaging method on the storage stability of poultry chicken ground meat stored in a high-O_2 gas (80% O_2 20% CO_2), modified atmosphere. The addition of rosemary slowed oxidation as compared to

ground meat without antioxidant addition (time zero storage ranged from 0.1 to 3.0 mg MDA/Kg sample).

The aim of this study was to evaluate the effects of rearing systems (free-range (FR) vs. conventional (C)) and packaging conditions (ordinary atmosphere (OA) vs. MAP (72% O_2 28% CO_2, modified atmosphere MA) on the oxidative stability of poultry thigh meat stored at 4°C for 14 days.

3.2 Materials and Method

Ammonium thiocyanate (NH_4SCN, $\geq$ 97.5%), barium chloride dehydrate ($BaCl_2 \cdot 2H_2O$, $\geq$ 99%), ethylenediamine-tetraacetic acid (EDTA) disodium salt (100%±1%) were supplied by Curtin Matheson Scientific Inc. Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, $\geq$ 99.0%), trichloroacetic acid (TCA) ($\geq$ 99%), ascorbic acid and double distilled water, were purchased from Carlo Erba Reagenti (Rodano, Italy). 2-Thiobarbituric acid (TBA) minimum 98%, sodium phosphate monobasic anhydrous (NaH_2PO_4), anhydrous sodium sulfate, potassium hydroxide were supplied by Sigma-Aldrich (St. Louis, USA). Sodium phosphate dibasic anhydrous (Na_2HPO_4) was purchased from Fisher Scientific (New Jersey, USA).

Chloroform, *n*-hexane, methanol and potassium chloride ($\geq$ 99%) were supplied by Merck (Darmstadt, Germany). Anhydrous sodium sulfate and potassium hydroxide were purchased from BDH (Poole, England) and Prolabo (Fontenay, France), respectively.

The phosphate buffer used for the TBARs determination, was prepared by adding 65.8 mL of 0.5 M NaH_2PO_4 and 111 mL of 0.5 M $Na_2HPO_4 \cdot H_2O$ (water solutions) in a 500 mL volumetric flask. pH was controlled, taken to neutrality (either with 1 N HCl or 1 N NaOH solutions), and made up to volume with water. To delay oxidation and prevent the pro-oxidative effect of

metals, proper amounts of EDTA and ascorbic acid were added to the phosphate buffer to reach a final concentration of 0.1% (w/v) of both of them.
To prepare the iron(II) chloride solution used for PV determination, 0.4 g barium chloride dihydrate were dissolved in 50 mL of water. This solution was slowly added and with constant stirring to an iron(II) sulfate solution (0.5 g FeS04·7H20 dissolved in 50 mL of water). Two milliliters of 10 N HCl solution was added to the resulting solution. The barium sulfate precipitate was filtered to give a clear iron(II) solution, which was stored in a brown bottle and kept in the dark. To prepare the ammonium thiocyanate solution, 30 g of ammonium thiocyanate was dissolved in water, and the volume was made up to 100 mL.

Experimental design

The study was carried out on 72 chickens, which are divided into two experimental groups. The first group (n=36) was composed by Ross 708 chickens (fast-growing genotype); which were reared indoor in a conventional (C) way, under intensive conditions; the birds were kept in a poultry house under controlled environmental conditions and without outdoor access. C female and male birds were separately raised for 39 and 50 d, respectively. Chicks were fed the same diet (0 to 14 d: starter; 15 to 35 d: grower; 36 to 50 d: finisher), as described in Table 10. The 2nd group (n=36) was composed by Hubbard Red (Isa) chickens (slow-growing genotype), which were kept outdoor under a free range (FR) system; FR female and male chickens were separately raised for 56 and 70 d, respectively.
Female and male chickens were slaughtered into 2 separate sessions, in order to obtain the main two commercial categories (rotisserie and cut-up, respectively).

Sample preparation

After slaughtering, 9 carcasses of each group (rotisserie and cut-up) were randomly selected and used to evaluate oxidation stability of thigh meat (with skin). Thighs were placed in polystyrene foam trays (18 x 13.5 x 2.5 cm) and either over-wrapped in polyethylene film (ordinary atmosphere, OA) or placed in a pouch made of polyethylene and polyamide injected with a modified gas mixture (72% O_2 28% CO_2, modified atmosphere MA). Four packed samples of each atmosphere type (time zero samples) (were immediately removed, minced and packed in plastic bags covered by aluminum foil under vacuum and kept at –18°C until analysis. The other packed samples were stored at 2-4°C for 14 days in the dark. Two packs of each atmosphere- rearing system combination were sampled every 3-4 days of storage (0, 3, 7, 10 and 14 d), minced and packed in plastic bags covered by aluminum foil under vacuum and kept at –18°C until analysis.

The experimental design is displayed in Table 11.

Table 10: Main ingredients and composition of experimental diets.

	Conventional diet			Free range diet			
	Starter	Grower	Finisher	Starter	Grower	Finisher	Finisher
	0-14 d	15-35 d	36- 50 d	0-14 d	15-35 d	36-54 d	54-70 d
Main ingredients (%)							
Corn	20.0	5.0	5.0	20.0	5.0	5.0	50.0
Wheat	35.8	57.0	59.9	39.5	60.9	65.8	
Soybean meal	29.7	24.1	21.8	22.6	20.3	16.6	
Sunflower meal				2.2			
Wheat bran	3.0	3.0	3.0	10.0	3.0	3.0	
Sulflower oil	5.0	7.1	7.0				
Soybean					3.0	3.0	
Soybean oil				1.5	3.7	3.3	
M2661							50.0
Calculated composition							
Energy (MJ of ME [a]/kg)	12.8	13.3	13.4	11.9	12.6	12.7	13.0
Dry matter (%)	88.2	88.4	88.3	87.7	88.1	88.1	87.8
Crude protein (%)	22.1	19.3	18.5	19.6	18.9	17.5	16.5
Lipid (%)	6.8	8.6	8.4	3.5	5.7	5.4	5.3
Crude fiber (%)	2.5	2.5	2.5	3.0	2.6	2.5	2.4
Ash (%)	5.5	4.8	4.4	5.6	5.1	4.4	4.5

[a] ME: Metabolizable Energy

Table 11: Experimental design.

	Conventional		Free range	
Trait	Thigh		Thigh	
Genotype	Ross 708		Isa	
Sex	Female	Male	Female	male
Stocking density (birds/sqm)	18	10	13	7
Outdoor access	-	-	From 28 to 56 d	From 28 to 70 d
Age at slaughter	39	50	56	70
Slaughter weight	1.9 kg	3.1 kg	1.8 kg	2.8 kg
Carcass yield	65 %	71 %	62 %	68 %
Carcass weight	1.2-1.3 kg	2.2-2.3 kg	1.1-1.2 kg	1.9-2.0 kg
Shelf-life study	0, 3, 7, 10, 14d	0, 3, 7, 10, 14d	0, 3, 7, 10, 14d	0, 3, 7, 10, 14d

Lipid extraction

Lipids were extracted according to a modified version (Boselli et al., 2001) of the method described by Folch et al. (1957). The frozen samples were minced and were homogenized (6575 g/rfc or 21500 rpm, for 3 min) 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 thermostated at 60°C for 20 min before adding 100 mL chloroform. After 2 min of homogenization (6575 g/rfc or 21500 rpm), the content of the bottle was filtered through filter paper to eliminate the solid residue, which consisted mostly of proteins. The filtrate was mixed thoroughly with 100 mL of a 1 M KCl solution and left overnight at 4°C in order to obtain phase separation. The lower phase containing the lipids was

collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Two lipid extractions were performed for each sample.

Spectrophotometic determination of peroxide value

PV was determined using a modified method of Shantha and Decker (1994). Briefly, 20 mg of extracted lipids were mixed with 9.8 mL of choloform:methanol (2:1, v/v) and 50 μL of thiocyanate/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 0.1-5 μg/mL ($y = 0.0311x - 0.0375$; $r^2 = 0.998$). Peroxide value was expressed as meq O_2/kg fat. Two replicates were run per sample.

Spectrophotometric determination of thiobarbituric reactive substances (TBARs)

TBARs were used as an indicator of the secondary lipid oxidation products using the method of Tarladgis et al. (1960). 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) trichloroacetic acid aqueous solution were then added and sample was homogenized followed by filtration. Five mL of 0.02 M aqueous solution of thiobarbituric acid were added to 5 mL of the resulted solution, in the capped tubes stored at 90°C for 20 min, followed by refrigeration. 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 0.03-2.26 μg mL^{-1} ($y = 0.0015x - 0.0078$; $r^2 = 0.999$). TBARs value was expressed as mg of MDA kg^{-1} of sample. Two replicates were run per sample.

Statistical analysis

Data were analyzed by means of one-way-analysis of variance ANOVA (GLM/PASW procedure) to test the effect of production system (conventional vs. free-range), atmosphere packaging (OA vs. MA) and shelf-life (0, 3, 7, 10, 14 days) on meat quality traits within each carcass category (rotisserie and cut-up). Overall differences between rearing means were tested according to Tukey's test, performed at 95% confidence level and considered to be significant when $P < 0.05$ (PASW Statistics, 17).

3.3 Results and Discussion

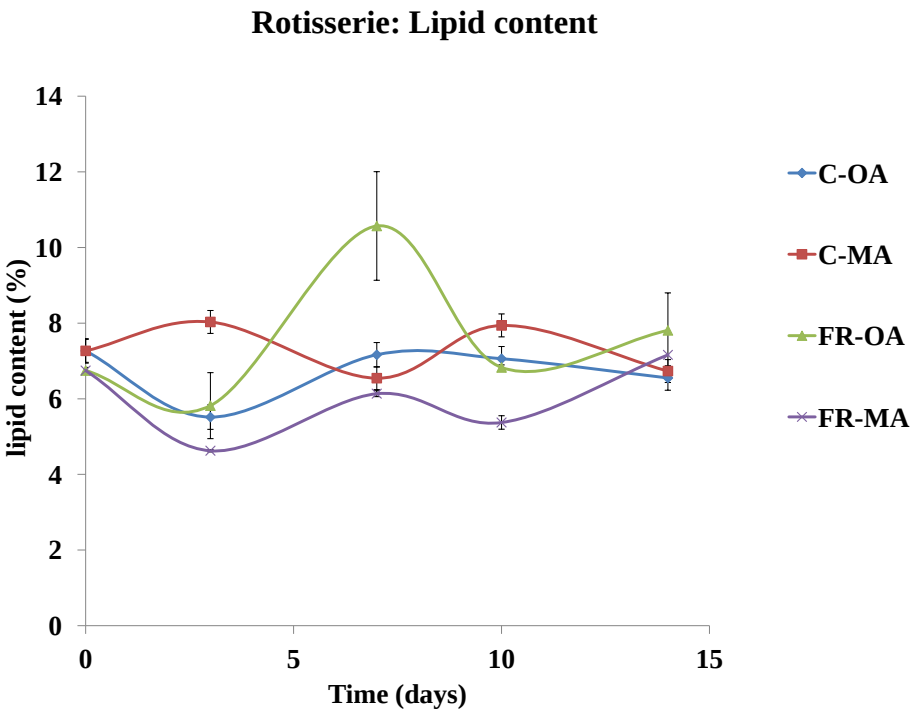
Lipid content

Figure 3 shows the effects of rearing systems on the average lipid content of thigh (with skin) meat. In the rotisserie and cut-up categories, the rearing system (C, FR), packaging atmosphere (OA, MA) and shelf-life (0, 3, 7, 10, 14 d) did not significantly affect the lipid content in all samples (5.5-10.7%).

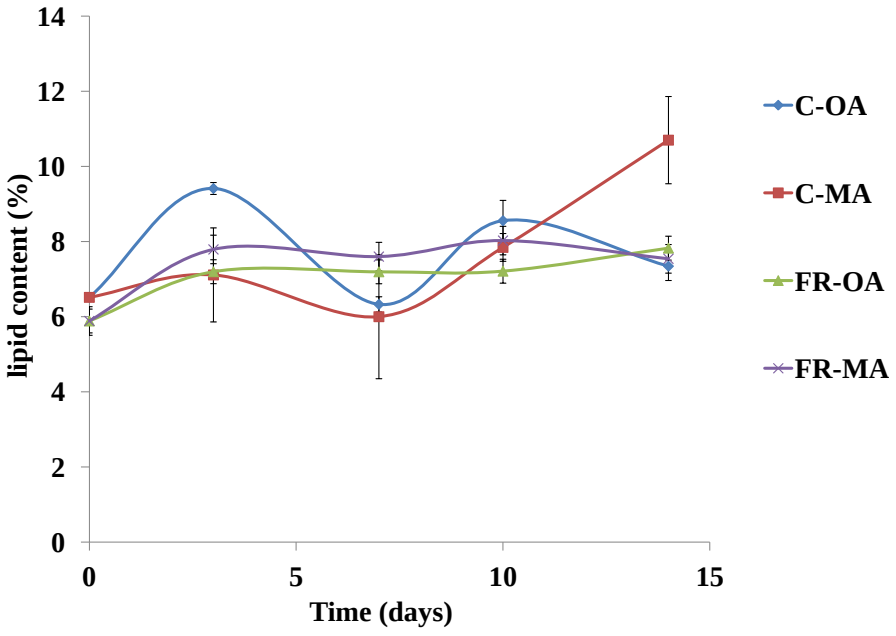
These results are similar than those reported in literature for stored poultry meat. In fact, Keokamnerd et al. (2008) reported 7.2% of lipid content in thigh meat (skinless). Variations on lipid content of chicken meat can be ascribed to growing seasons, diet, sex, animal genetics, breeding system, and analytical methodology.

Figure 3: Lipid content of thigh meat of rotisserie (A) and cut-up (B) carcasses from conventional and free range production systems (mean ± ds). Each value is the average of two independent replicates for each point. ***ns = P > 0.05***

(A)



(B)



Lipid oxidation

Lipid oxidation of poultry meat obtained with different rearing systems and packaging conditions was evaluated by PV and TBARs. Figures 4 and 5 show the effects of rearing on the PV and TBARs of thigh meat of both rotisserie and cut-up categories. In general, a low lipid oxidation level was observed in all samples, which was confirmed by both primary and secondary oxidation products parameters; these data are in agreement with those reported in literature (Keokamnerd et al., 2008). PV ranged from 0.50 to 11.46 and from 0.47 to 8.28 meq O_2/kg of lipid in rotisserie and cut-up thigh meat, respectively, being below the PV level (20 meq O_2/kg lipids) associated with oil rancidity. No significant effects ($P > 0.05$) of rearing system (C, FR), packaging conditions (O, M) and shelf-life (0-14 d) on PV, were found. However, it is possible to observe some trends in the formation of primary oxidation products in both categories (Figures 4A and 5A). In fact, in the rotisserie FR-OA and FR-MA samples, a non-significant decrease of PV was observed at 10 and 14 days of storage, with respect to those found at shorter storage times (3 and 7 days).

In addition, storage for 10 and 14 days led to a non-significant PV increase in cut-up category, with the highest PV in FR-M.

Regarding secondary oxidation products, TBARs ranged from 0.11 to 1.24 and from 0.08 to 0.70 mg MDA/kg of sample in rotisserie and cut-up thigh meat, respectively, being below the TBARs level (1 mg MDA/kg of sample) associated with lamb meat rancidity (Ripoll et al., 2011); the limiting threshold for oxidized meat acceptability varies according to the animal and to the study. The statistical analysis shows some significant differences ($P < 0.05$) due to rearing system and packaging conditions as TBARs content was higher in rotisserie category, breeded with a free-range system and stored

under ordinary atmosphere. Both commercial categories displayed a significant increase in TBARs in samples stored for 10 and 14 days with respect to those stored from 0 to 7 days. The higher TBARs formation rate in samples with long shelf-life is due to hydroperoxide breakdown, which are converted into secondary oxidation products, such as aldehydes. PV and TBARs data obtained in the present work were lower than those found in poultry meat (neck and frame meat) obtained by a control diet (PV= 2.50-38.34 meq O_2/kg lipid and TBARs 0.57-4.90 mg MDA/kg meat) (Ozkececi et al., 2008) and those detected in fresh chicken breast meat (skinless and boneless fillet) also produced with a control diet (TBARs= 0.50 mg MDA/kg meat) (Petrou et al., 2012).

TBARs data obtained in the present work were lower than those found in poultry meat by Smiddy et al. (2002). They investigated the use of oxygen sensors for the non-destructive measurement of the oxygen content in modified atmosphere and vacuum packs of cooked chicken patties, investigating the impact of oxygen content on lipid oxidation. Samples were packaged under vacuum and modified atmosphere (70% N_2 30% CO_2) and stored at 4 °C, under fluorescent light, for 25 days. MAP chicken samples were oxidized to a greater extent than vacuum packaged meat ($P < 0.05$). Lipid oxidation in MAP samples increased significantly ($P < 0.01$) over time, giving TBARS values between 6.25 and 11.62 mg MDA/kg meat by day 25.

These differences on oxidative stability could be ascribed to several factors, such as chicken characteristics (breed, sex, age), feeding (composition, amount and time of supplementation), slaughtering conditions, postmortem history, sample preparation and storage/packaging conditions. To prevent the development and spread of spoilage and pathogenic microorganisms, to extend shelf-life of meat products bioactive packaging technologies could be a potential alternative solution (Coma, 2008). O_2-scavenging technology may be used appropriately to remove residual O_2 after MAP or vacuum packaging

and to absorb O_2 that permeates through the packaging film, thus helping photo-oxidation prevention.

Correlations between oxidation parameters of chicken meat

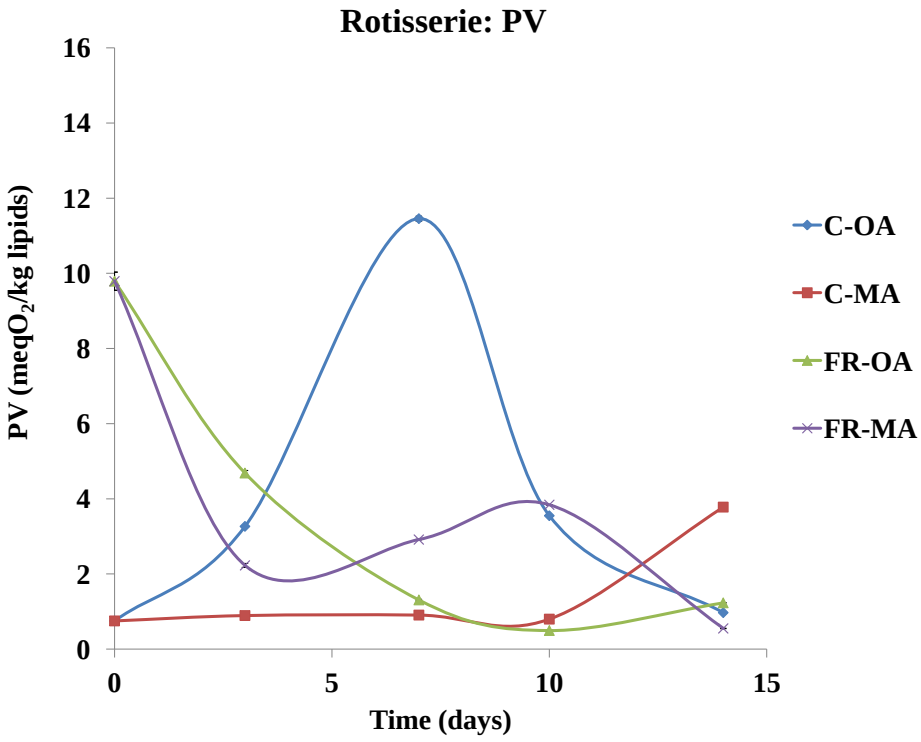
A correlation study (Spearman test, α= 0.05) was performed on the results obtained for fatty acid composition and the oxidation parameters of poultry meat obtained with the two types of rearing systems. For a better data comprehension, only significant correlations are here discussed.

A positive, linear correlation was only found between PV and TBARs in rotisserie thigh packaged under MA atmosphere (r= 0.503, P= 0.05*), which supports the well-known interdependence between hydroperoxides and their demolition/evolution in secondary oxidation products. The modified atmosphere, in fact, allowed to visualize the chemical PV formation and its conversion into TBARs, as this packaging system limited the contribution of microorganism growth and their impact on lipid oxidation through enzymatic mechanisms.

Moreover, a positive linear correlation was found between PV and TBARs in cut-up thigh produced by FR (r= 0.606, P= 0.01**) and by C system (r= 0.705, P= 0.01**). This means that both rearing systems did not affect the interdependence between hydroperoxides and their conversion into secondary oxidation products.

Figure 4: PV (A) and TBARs (B) of thigh meat of rotisserie carcasses from conventional and free range production systems (mean ± ds). Each value is the average of four analytical replicates for each point. *ns* = *P> 0.05, * P<0.05*.

(A)



(B)

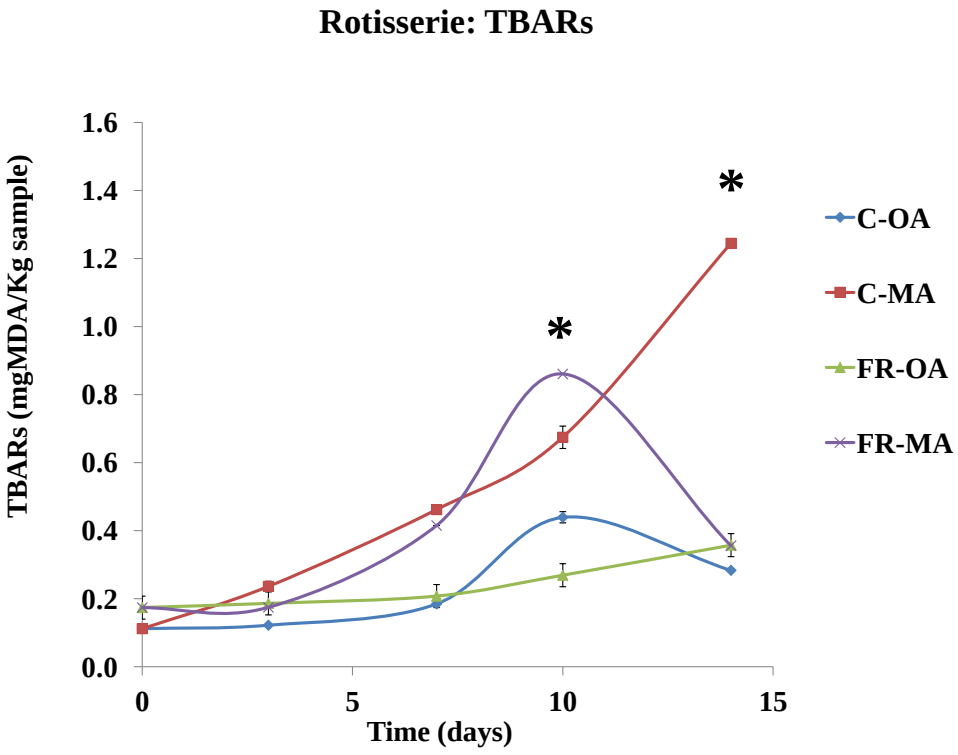
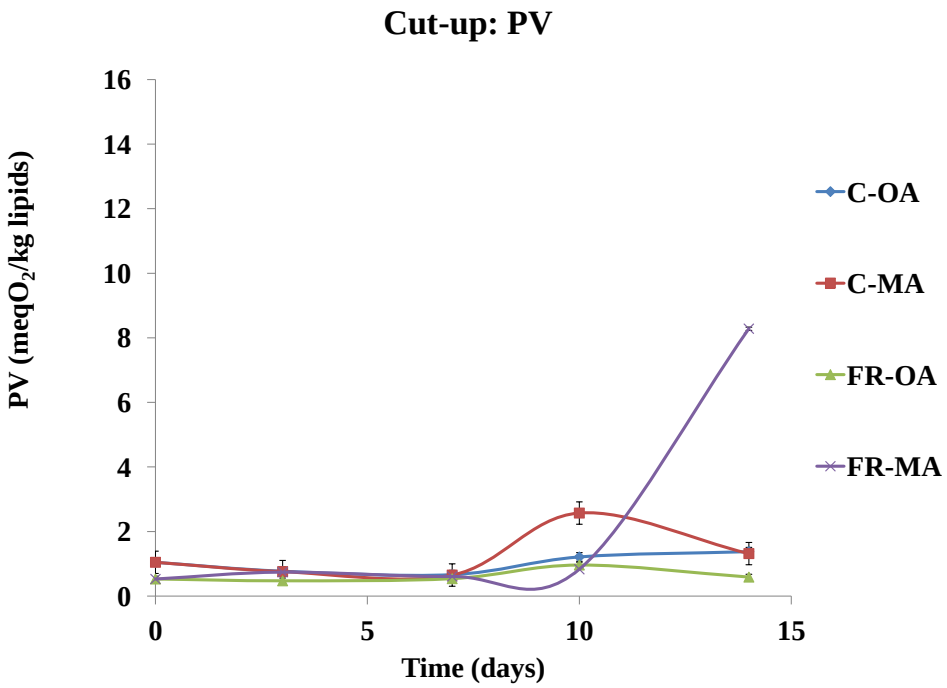
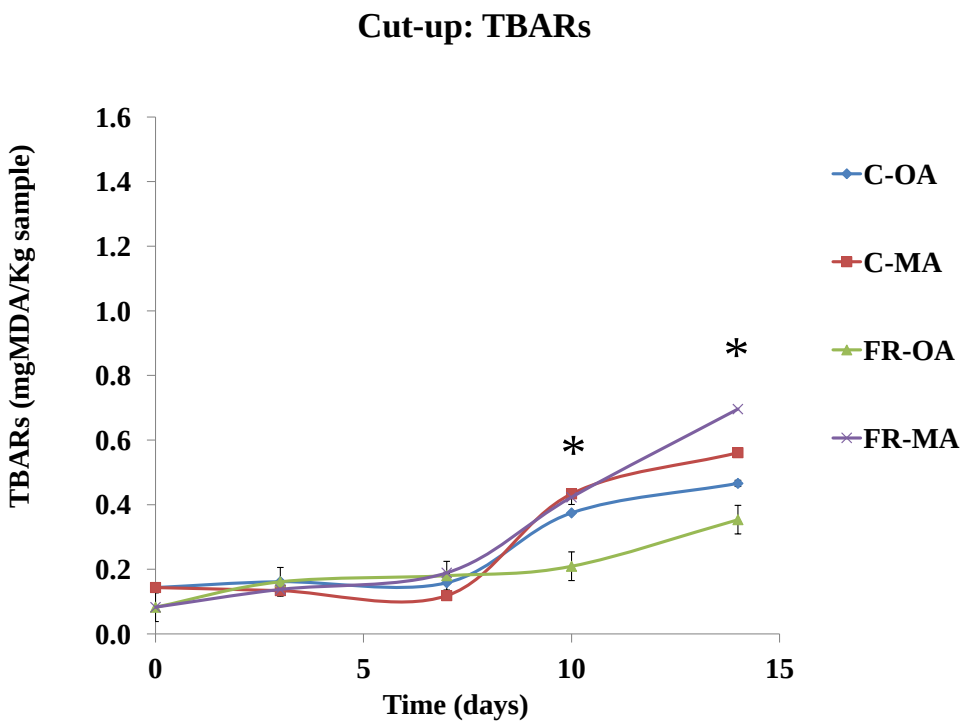


Figure 5: PV (A) and TBARs (B) of thigh meat of cut-up carcasses from conventional and free range production systems (mean ± ds). Each value is the average of four analytical replicates for each point. *ns* = *P>0.05*, * *P<0.05*.

(A)



(B)



Conclusions

In general, a low level of both primary and secondary oxidation products was found in all chicken thigh meat samples obtained with two rearing systems (free-range (FR) vs. conventional (C)) and stored at 4°C for 14 days, using two packaging conditions (ordinary atmosphere (OA) vs. MAP (72% O_2 28% CO_2, modified atmosphere MA). Although the overall primary oxidation level of poultry meat was not significantly affected by rearing system, packaging conditions and storage time, secondary oxidation showed a significant increase in rotisserie category, obtained with FR breeding system and stored under ordinary packaging atmosphere. Moreover, long storage (10 and 14 days) would not be advisable under the tested conditions, as both commercial categories exhibited a significant TBARs increase. Therefore, it can be concluded that the two different rearing systems, in combination with ordinary or modified atmosphere packaging, led to a low oxidation level in poultry meat, but it is anyway advisable to avoid long-storage periods (over 7 days) under these conditions to ensure meat overall quality and consumer's acceptability.

4 Effects of feed supplementation with thymol, packaging and storage conditions on lipid oxidation of poultry meat

4.1 Abstract

The aim of this study was to evaluate the effects of thymol supplementation and packaging conditions on the oxidative stability of poultry thigh meat stored at 4°C for 14 days. Male birds were a fast growing hybrid (Ross 708) and were raised for 42 d with a conventional (C) rearing system. A total of 81 Ross 708 chickens were homogeneously allotted to three experimental groups: A group (control diet containing corn/wheat/soybean without thymol addition), E group (control diet supplemented with 0.1% of thymol and F group (control diet supplemented with 0.2% of thymol). Chickens were slaughtered to obtain the cut-up commercial category. After slaughtering, 27 carcasses of each group were randomly selected and stored with two different packaging atmosphere (ordinary (O) and modified (M) atmosphere). Lipid content, peroxide value (PV) and thiobarbituric acid reactive substances (TBARs) were determined. Packaging atmosphere (O, and M) and diet (A, E and F) did not significantly affect the lipid content of thigh meat (A=11.8-18.2%; E=12.4-18.6%; F=12.0-16.2%). Furthermore, the diet did not significantly affect PV (A= 0.47-12.5 meq O_2/kg lipids; E= 0.72-12.9 meq O_2/kg lipids; F= 0.99-11.0 meq O_2/kg lipids) and TBARs (A= 0.14-0.73 mg MDA/kg sample; E=0.11-0.56 mg MDA/kg sample; F=0.17-0.49 mg MDA/kg sample). Packaging with ordinary (OA) and modified atmosphere (MA, 72% O_2 28% CO_2) significantly ($p<0.05$) influenced the level of primary oxidation (O 0.47-12.99 meq O_2/kg lipids; M 1.12-12.45 meq O_2/kg lipids). However, no significant effects of the packaging atmosphere were observed on TBARs (O 0.11-0.56 mg MDA/kg sample; M 0.11-0.73 mg MDA/kg sample). In conclusion, dietary supplementation with thymol extract at the tested levels did not provide further protection against lipid oxidation to

poultry thigh meat stored under diverse packaging atmosphere (ordinary and modified atmosphere (MA, 72% O_2 28% CO_2).

4.1 Introduction

Over the past few years, food product development have focused on the utilization of natural additives/ingredients instead of synthetic ones, as a consequence of market trends and consumer's requests. The potential beneficial properties of natural antioxidants have encouraged researchers to further investigate on this field. Herbs and spices have traditionally employed for their flavor-enhancement characteristics and their medicinal properties. Due to the growing occurrence of chronic diseases worldwide and the corresponding rise in health care costs, there is an increasing interest on the multiple health benefits of herbs and spices, including cancer risk reduction (Kaefer et al., 2008). Several studies have demonstrated the beneficial preventive anticancer effects of natural green tea polyphenols on human cancer cells and their potential antitumor properties (Chen et al., 2011). These natural bioactive compounds (like biophenols and flavonoids) possess a variety of beneficial effects including antioxidant and anticarcinogenic activities, protection against coronary diseases as well as antimicrobial properties (Boros et al., 2010). Herbs and spices rich in these bioactive compounds can be particularly helpful for controlling lipid oxidation in meat during processing and storage, and thus for reducing potential changes on the quality, nutritional and sensory profile of the product. Antioxidants can be added either by dietary supplementation or directly to the product as herbs or solutions. Among the latter, thymus species have been reported as sources of different phenolic acids and flavonoids (Vila, 2002). The antioxidant activities of various solvent extracts and essential oil composition of some *Thymus* species have been recently evaluated (Ozen et al., 2011). A

significant antioxidant effect of thyme oil on the preservation of vacuum-packaged chicken liver has been recently reported (Papazoglou et al., 2012). One of the bioactive components of thyme is thymol, which is a molecule that have intrinsic bioactivities on animal physiology and metabolism. Reiner et al. (2009) have shown that thymol acts on the GABAA receptor in phospholipid liposomes. In fact, thymol has wide inhibitory effects against foodborne pathogens, including *Salmonella* on chicken breast (Lu et al., 2012). Thymol extract in combination with carvacrol and grapefruit seed extract (GFSE) (0-300 mg/L), can also retard the oxidation process by maintaining MDA values below 2 mg/kg sample in poultry meat patties added directly with antioxidants (Lucera et al., 2009). However, Luna et al. (2010) did not found significant effects on breast chicken oxidation, when feed supplementation with thymol and its isomer carvacrol were used.

The aim of this study was to evaluate the effects of thymol supplementation and packaging conditions (ordinary vs. modified atmosphere) on the oxidative stability of poultry thigh meat stored at 4°C for 14 days.

4.2 Materials and Methods

Solvents and reagents

Ammonium thiocyanate (NH_4SCN, $\geq$ 97.5%), barium chloride dehydrate ($BaCl_2 \cdot 2H_2O$, $\geq$ 99%), ethylenediamine-tetraacetic acid (EDTA) disodium salt (100%±1%) were supplied by Curtin Matheson Scientific Inc. Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, $\geq$ 99.0%), trichloroacetic acid (TCA) ($\geq$ 99%), ascorbic acid and double distilled water, were purchased from Carlo Erba Reagenti (Rodano, Italy). 2-Thiobarbituric acid (TBA) minimum 98%, sodium phosphate monobasic anhydrous (NaH_2PO_4), anhydrous sodium sulfate and potassium hydroxide were supplied by Sigma-Aldrich (St. Louis,

USA). Sodium phosphate dibasic anhydrous (Na_2HPO_4) was purchased from Fisher Scientific (New Jersey, USA).

Chloroform, *n*-hexane, methanol and potassium chloride (≥ 99%) were supplied by Merck (Darmstadt, Germany). Anhydrous sodium sulfate and potassium hydroxide were purchased from BDH (Poole, England) and Prolabo (Fontenay, France), respectively.

The phosphate buffer used for the TBARs determination, was prepared by adding 65.8 mL of 0.5 M NaH_2PO_4 and 111 mL of 0.5 M $Na_2HPO_4 \cdot H_2O$ (water solutions) in a 500 mL volumetric flask. pH was controlled, taken to neutrality (either with 1 N HCl or 1 N NaOH solutions), and made up to volume with water. To delay oxidation and prevent the pro-oxidative effect of metals, proper amounts of EDTA and ascorbic acid were added to the phosphate buffer to reach a final concentration of 0.1% (w/v) of both of them.

To prepare the iron(II) chloride solution used for PV determination, 0.4 g barium chloride dihydrate were dissolved in 50 mL of water. This solution was slowly added and with constant stirring to an iron(II) sulfate solution (0.5 g FeS04·7H20 dissolved in 50 mL of water). Two milliliters of 10 N HCl solution was added to the resulting solution. The barium sulfate precipitate was filtered to give a clear iron(II) solution, which was stored in a brown bottle and kept in the dark. To prepare the ammonium thiocyanate solution, 30 g of ammonium thiocyanate was dissolved in water, and the volume was made up to 100 mL.

Experimental Design

This study was carried out on 81 Ross 708 male chickens (fast-growing genotype), which were reared indoor in a conventional way, under intensive conditions (Table 12). The birds were kept in a poultry house under controlled environmental conditions and without outdoor access. Chickens were homogeneously allotted into three experimental groups:

1) A group (control diet containing corn/wheat/soybean without thymol addition);

2) E group (control diet supplemented with 0.1% of thymol);

3) F group (control diet supplemented with 0.2% of thymol).

Chicks were raised for 42 d and were fed the same control diet (0 to 21 d: starter; 22 to 36 d: grower; 37 to 42 d: finisher; Table 13) plus thymol supplementation for E and F groups. An extract of thymol (≥ 50%) was used for this experimentation (provided by Ernesto Ventòs S.A., Barcelona, Spain). Chickens were slaughtered to obtain the cut-up commercial categories.

Preparation of samples

After slaughtering, 81 carcasses were randomly selected and used to evaluate oxidation stability of thigh meat (with skin). Thighs were placed in polystyrene foam trays (18 x 13.5 x 2.5 cm) and either over-wrapped in polyethylene film (ordinary atmosphere, OA) or placed in a pouch made of polyethylene and polyamide injected with a modified gas mixture (72% O_2 28% CO_2 modified atmosphere MA). Three packed control samples of each diet group A, E and F (time zero samples) were immediately removed from the carcass, minced, packed in plastic bags covered by aluminum foil under vacuum and kept at –18°C until analysis.

The other packed samples were stored at 2-4°C for 14 days in the dark. Three packs of each atmosphere-diet group were sampled every 3-4 days of storage (0, 2, 5, 10 and 14 d), minced and packed in plastic bags covered by aluminum foil under vacuum and kept at –18°C until analysis.

The experimental sampling design is displayed in Figure 6.

Table 12: Experimental design

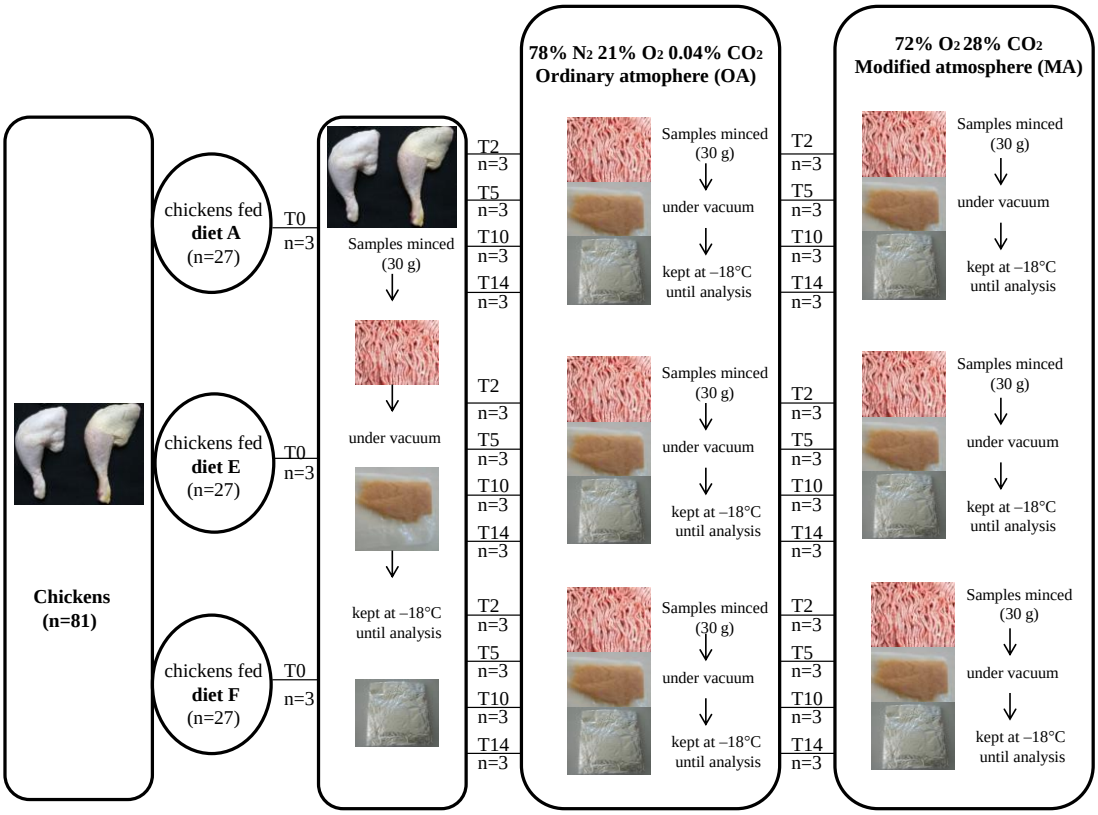
	Conventional
Trait	Thigh
Genotype	Ross 708
Sex	Male
Stocking density (birds/sqm)	10
Outdoor access	-
Age at slaughter	50
Slaughter weight	3.1 kg
Carcass yield	71 %
Carcass weight	2.2-2.3 kg
Shelf-life	0, 2, 5, 10, 14 d

Table 13: Ingredients and nutrient composition of the basal diet.

	Diet (A group)		
Main ingredients (%)	**Starter (0-21 d)**	**Grower (22-36 d)**	**Finisher (37-42 d)**
Wheat meal	25.00	39.98	40.89
Corn meal	26.50	5.00	5.00
White Corn meal	5.00	8.00	8.00
Sorghum meal	0.00	10.00	12.00
Soybean meal	22.66	18.57	16.35
Soybean bran meal	10.00	10.00	10.00
Vegetable oil	3.14	4.47	4.27
Corn glutin	3.00	0.00	0.00
Vitamin supplement	0.46	0.40	0.26
Dicalcium phosphate	2.06	1.57	1.27
Calcium carbonate	0.92	0.83	0.81
Sodium Chloride	0.25	0.21	0.22
Baking soda	0.10	0.15	0.15
Lysine	0.42	0.34	0.35
Methionine	0.31	0.29	0.28
Choline	0.10	0.10	0.05
Threonine	0.08	0.08	0.10
Calculated composition			
Energy (MJ of ME [a]/kg)	12.78	13.14	13.25
Dry matter (%)	88.62	88.66	88.58
Crude protein (%)	22.08	19.42	18.62
Lipid (%)	6.87	7.97	7.81
Crude fiber (%)	2.71	2.65	2.62
Ash (%)	5.96	5.26	4.86

[a] ME: Metabolizable Energy

Figure 6: Sampling design for experiment.



Lipid extraction

Lipids were extracted according to a modified version (Boselli et al., 2001) of the method described by Folch et al. (1957). The frozen samples were minced and were homogenized (6575 g/rfc or 21500 rpm, for 3 min) 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 thermostated at 60°C for 20 min before adding 100 mL chloroform. After 2 min of homogenization (6575 g/rfc or 21500 rpm), the content of the bottle was filtered through filter paper to eliminate the solid residue, which consisted mostly of proteins. The filtrate was mixed thoroughly with 100 mL of a 1 M KCl solution and left overnight at 4°C in order to obtain phase separation. The lower phase containing the lipids was collected and dried with a rotary evaporator. The fat content was determined gravimetrically. Two lipid extractions were performed for each sample.

Spectrophotometric determination of peroxide value

PV was determined using a modified method of Shantha and Decker (1994). Briefly, 20 mg of extracted lipids were mixed with 9.8 mL of choloform:methanol (2:1, v/v) and 50 μL of thiocyanate/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 0.1-5 μg/mL ($y = 0.0311x - 0.0375$; $r^2 = 0.998$). Peroxide value was expressed as meq O_2/kg fat. Two replicates were run per sample.

Spectrophotometric determination of thiobarbituric reactive substances (TBARs)

TBARs were used as an indicator of the secondary lipid oxidation products using the method of Tarladgis et al. (1960). 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) trichloroacetic acid aqueous solution were then added and sample was homogenized followed by filtration. Five mL of 0.02 M aqueous solution of thiobarbituric acid were added to 5 mL of the resulted solution, in the capped tubes stored at 90°C for 20 min, followed by refrigeration. 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 0.03-2.26 μg mL^{-1} ($y = 0.0015x - 0.0078$; $r^2 = 0.999$). TBARs value was expressed as mg of MDA kg^{-1} of sample. Two replicates were run per sample.

Statistical analysis

Data were analyzed by means of one-way-analysis of variance ANOVA (GLM/PASW procedure) to test the effect of the diet (A, E, and F group), atmosphere packaging (OA vs. MA) and storage (0, 2, 5, 10 and 14 d) on meat quality traits in cut-up tight. Overall differences between rearing means were tested according to Tukey's test, performed at 95% confidence level and considered to be significant when $P < 0.05$ (PASW Statistics, 17).

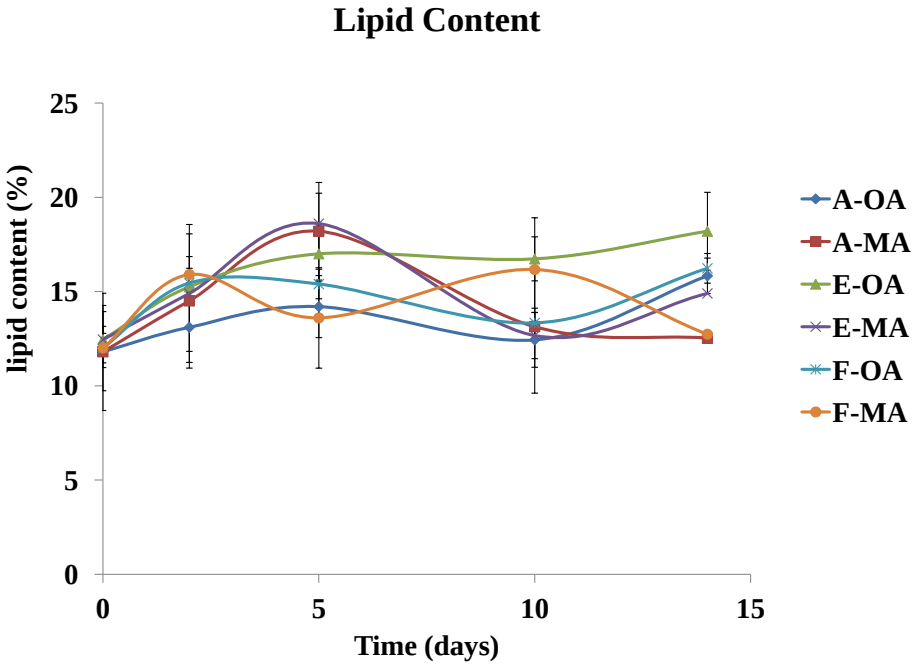
4.3 Results and Discussion

Lipid content

Figure 7 shows the effects of diet on the average lipid content of thigh (with skin) meat stored under different packaging conditions. The packaging atmosphere (O, and M) and the diet (A, E and F) did not significantly affect

the lipid content of thigh meat (A=11.8-18.2%; E=12.4-18.6%; F=12.0-16.2%).

Figure 7: Lipid content of cut-up thigh meat obtained with thymol dietary supplementation and a conventional production system (mean ± ds). Each value is the average of three independent replicates. *ns* = *P*> *0.05*



Lipid oxidation

Lipid oxidation of poultry meat obtained with different diets and packaging systems was evaluated by PV and TBARs. Figures 8A and 8B show the PV and TBARs trends of thigh meat produced with different dietary supplementation, during storage with diverse packaging. In general, a low lipid oxidation level was observed in all samples, which was confirmed by the both primary and secondary oxidation products parameters; these data are in agreement with those reported in literature (Luna et al., 2010). PV and TBARs ranged from 0.47 to 12.9 meq O_2/kg of lipids and from 0.11 to 0.73

mg MDA/kg of sample, respectively, being below the PV (20 meq O_2/kg lipids) and TBARs levels (1 mg MDA/kg of sample; Ripoll et al., 2011) associated with lamb meat rancidity. The limiting threshold for oxidized meat acceptability varies according to the animal and to the study. However, the effect of thymol supplementation on the lipid oxidation of chicken thigh samples was ineffective. In fact, diet did not significantly affect PV level (A= 0.47-12.5 meq O_2/kg lipids; E= 0.72-12.9 meq O_2/kg lipids; F= 0.99-11.0 meq O_2/kg lipids) and TBARs content (A= 0.14-0.73 mg MDA/kg sample; E=0.11-0.56 mg MDA/kg sample; F=0.17-0.49 mg MDA/kg sample). It might be possible that the purity of thymol extract and/or the supplementation level used were not enough to display an antioxidant activity. It is also possible that the antioxidant activity of thymol becomes more evident if combined with other bioactive compounds (like carvacrol) due to a synergic effect, as demonstrated by Lucera et al. (2010). The direct addition of thyme to meat products may be another alternative that could lead to better oxidative stability than feeding supplementation of the thymol extract; however, its impact on the sensory profile of poultry and consumer's acceptance should be verified.

On the contrary, the packaging atmosphere (O, M) significantly ($P \leq 0,05$) influenced the level of primary oxidation (O 0.47-12.9 meq O_2/kg lipids; M 1.12-12.5 meq O_2/kg lipids). However, no significant effects of the packaging atmosphere were observed on TBARs (O 0.11-0.56 mg MDA/kg sample; M 0.11-0.73 mg MDA/kg sample). The susceptibility of broiler thigh to the primary lipid oxidation as a function of storage time and feed supplementation is quite fluctuating as illustrated in Figure 8A. However, it is possible to observe some trends. As expected, when shelf-life days increased (14 d), PV was significantly higher as compared with those of samples stored for few days (2 and 5d) in A-MA and F-MA, respectively. All other groups had an opposite primary oxidative trend. The A-OA group (control diet stored

with ordinary atmosphere) exhibited the lowest PV value as compared to the other groups. Regarding secondary oxidation products, the statistical analysis shows some significant differences ($P<0.05$) on TBARs as related to storage time, in fact, all samples (A-OA, A-MA, E-OA, E-MA, F-OA, F-MA) displayed a TBARs increase from 5 to 14 days. The higher TBARs formation rate in samples with longer storage time is due to hydroperoxide breakdown, which are converted into secondary oxidation products, such as aldehydes.

Luna et al. (2010) evaluated the effects of thymol and its isomer carvacrol (150 mg/kg of each compound) on lipid oxidation when supplemented to feed, using an extract with a 97% of purity. TBARs detected were very low (0.00008-0.00018 mg MDA/kg meat) and the authors observed that, after 10 d of storage, TBARs values of thigh samples from the control group were significantly higher ($P < 0.001$) than those found in the enriched group. TBARs data obtained by Luna et al. (2010) were lower than those found in the present work; these differences on oxidative stability could be ascribed to several factors, such as chicken characteristics (breed, sex, age), feeding (composition, extract purity, amount and time of extract supplementation), slaughtering conditions, postmortem history, sample preparation and storage conditions.

Kamboh et al. (2013) investigated the effects of dietary supplementation of purified bioflavonoids (genistein and hesperidin at 5 and 20 mg per kg of feed, respectively) on the antioxidant status of broilers, as potential alternatives to plant/herbs or synthetic antioxidants. The output of this research study confirmed that dietary bioflavonoids genistein and hesperidin could positively improve the total antioxidant capacity of plasma in broilers , that have been fed with bioflavonoid-supplemented diets. Thus, bioflavonoids could be a feasible alternative for feeding supplementation to the produce healthier chicken meat. Botsoglou et al. (2002) demonstrated the efficacy of oregano essential oil (50 and 100 mg/kg of feed) on the performance of

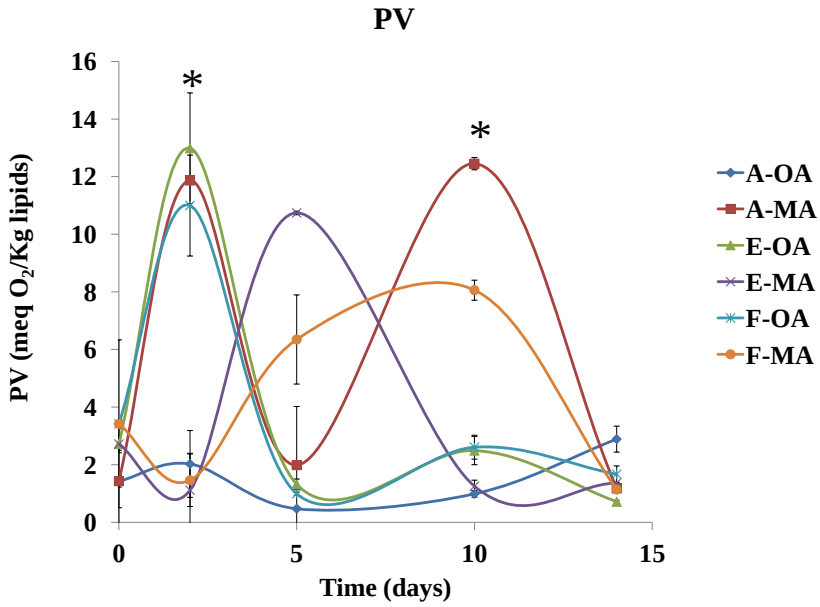
broilers, and the susceptibility of the resulting broiler meat to iron-induced lipid oxidation. Iron-induced lipid oxidation showed that as oregano oil increased in the diet, malonylaldehyde values decreased in tissue samples, suggesting that the oil, particularly at 100 mg/kg of feed, exerted an antioxidant effect on chicken tissues. All these results demonstrated the importance of bioactive compounds purity and the great potentiality of their utilization in combination, as some of them exhibit synergic effects.

Correlations between oxidation parameters of chicken meat

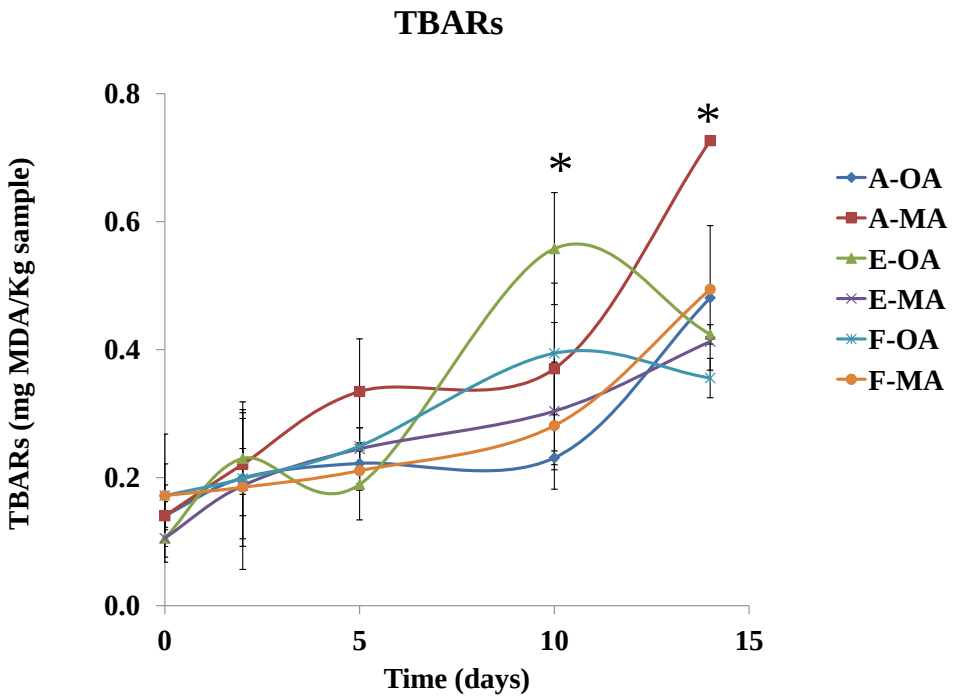
A correlation study (Spearman test, α= 0.05) was performed on the results obtained for the oxidation parameters of poultry meat obtained with the two types of packaging (AO, MA) and three diets (A, E, F). No significant correlations between the oxidation parameters were found, though.

Figure 8: PV (A) and TBARs (B) of cut-up thigh meat obtained with thymol dietary supplementation and a conventional production system (mean ± ds). Each value is the average of three independent replicates. *$P<0.05$ ns = $P > 0.05$

(A)



(B)



Conclusions

In general, a low level of both primary and secondary oxidation products was found in all chicken thigh meat samples obtained with different diets and packaging conditions during storage at 4°C for 14 days. Packaging atmosphere (OA, and MA) and diet (A, E and F) did not significantly affect the lipid content. Packaging with ordinary (OA) and modified atmosphere (MA, 72% O_2 28% CO_2) significantly ($p<0.05$) influenced the level of primary oxidation (O 0.47-12.99 meq O_2/kg lipid; M 1.12-12.45 meq O_2/kg lipid). However, no significant effects of the packaging atmosphere were observed on TBARs. Regarding secondary oxidation products, the statistical analysis shows some significant differences ($P<0.05$) on TBARs as related to storage time, in fact, all samples displayed a TBARs increase from 5 to 14 days. In conclusion, dietary supplementation with thymol extract at the tested levels did not provide further protection against lipid oxidation to poultry thigh meat stored under diverse packaging atmosphere (ordinary and modified atmosphere (MA, 72% O_2 28% CO_2).

5 Health-promoting effects of bioactive food components: individual and combinatorial effects on the inhibition of inflammation

5.1 Abstract

Inflammation is associated with cancer and other chronic diseases. Many dietary bioactive compounds have been identified as potential anti-inflammatory agents, but their mechanisms of action have not been fully investigated. In the present work, the individual and combinatorial effects of several dietary food components (thymol (THY), polymethoxyflavones (PMFs), curcumin derivates (CURC), luteolin (LUT), sulforaphane (SFN), and docosa-4,7,10,13,16,19-hexaenoic acid (DHA)) against lipopolysaccharide (LPS)-induced acute inflammation were evaluated, with emphasis on the role of anti-inflammatory (heme oxygenase-1 (HO-1)) and pro-inflammatory enzymes (inducible nitric oxide (iNOS) and cyclooxygenase2 (COX-2)). All compounds showed individual anti-inflammatory effects but only LUT+TAN and LUT+SFN, had strong synergic effects. Noncytotoxic concentrations of LUT (3-15 μM), TAN (6-30 μM), SFN (0.24-1.2 μM) and their combinations were studied in LPS-stimulated RAW 264.7 macrophage cells. The results showed that both combination (LUT + TAN; LUT + SFN) treatments produced much stronger inhibitory effects on the production of nitric oxide (NO) than those compounds individually. However, high concentrations of LUT were also able to inhibit inflammation. These enhanced inhibitory effects were synergistic, according to the isobologram analysis. Western blot analysis showed that combined LUT and TAN pretreatments synergistically decreased LPS-induced COX-2 and iNOS protein expression levels and increased HO-1 protein expression. LUT and SFN combination had the same results, as they synergistically decreased iNOS protein expression levels and increased both HO-1 and COX-

2 proteins expression. Enzyme-linked immunosorbent assay (ELISA) indicated that low doses of combined LUT and TAN significantly suppressed LPS-induced upregulation of IL-1 (Interleukin-1), IL-6 (Interleukin-6), and PGE2 (Prostaglandin E2). Real-time polymerase chain reaction analysis indicated that low doses of combined LUT and TAN significantly suppressed LPS-induced upregulation of IL-1, IL-6, COX-2, and iNOS mRNA levels and synergistically increased HO-1 mRNA levels. These anti-inflammatory effects of both combination treatments (LUT + TAN; LUT + SFN) were stronger than those produced by the single compounds. Overall, our results demonstrated for the first time that both combination (LUT + TAN; LUT + SFN) produced synergistic anti-inflammatory effects in LPS-stimulated macrophage cells.

5.2 Introduction

Cancer is a major disease worldwide and is the leading cause of death in the World. It is one disease that fits the paradigm “the more we know, the less we understand its intricacies” (Aggarwal et al., 2009). It has been reported that only 5–10% of all cancer cases can be attributed to genetic defects, whereas the remaining 90–95% have their roots in the environment and lifestyle. The evidence indicates that of all cancer-related deaths, almost 25–30% are due to tobacco, as many as 30–35% are linked to diet, about 15–20% are due to infections, and the remaining percentage are related to other factors like radiation, stress, physical activity, environmental pollutants, etc. (Anand et al., 2008). Therefore, most of cancer cases might be preventable by dietary modification, depending on the dietary components. The European Prospective Investigation into Cancer and Nutrition (EPIC) was specifically designed to investigate the relationship between diet and cancer and other chronic diseases, with the aim of making a significant contribution to the

accumulated scientific knowledge by overcoming limitations of previous studies (Gonzalez et al., 2010). Epidemiological evidence has consistently indicated that diet abundant in fruits and vegetables may reduce the risk of cancer, and this effect has been attributed to the presence of bioactive components in these foods. Herbs also exhibit chemopreventive potential, and several studies have shown possible anticancer effects (Vainio et al., 2006; Liu, 2003). Many studies have therefore focused on the identification of beneficial effects of dietary bioactive food components to develop new suitable strategies for long-term promotion of human health, in particular against cancer and inflammation (Wong et al., 2008; Demaria et al., 2010; Hämäläinen et al., 2011; Murakami et al., 2012). Considering these potential effects, dietary bioactive food components have been added to feeding to improve oxidative and microbial stability of food products from animal origin (Arihara, 2006; Coma, 2008; Leusink et al., 2010; Zhang et al., 2010).

Inflammation is associated with cancer and other chronic diseases, therefore inflammatory cells and soluble factors are present in many tumors (Demaria et al., 2010). The role of inflammation in cancer was first proposed by Rudolf Virchow in 1863, when he observed the presence of leukocytes in neoplastic tissue (Aggarwal et al., 2009). In 2000, Hanahan and Weinberg proposed a model to define the six properties that a tumor acquires: unlimited replicative potential, ability to develop blood vessels (angiogenesis), evasion of programmed cell death (apoptosis), self-sufficiency in growth signals, insensitivity to growth inhibitors, tissue invasion and metastasis (Mantovani et al., 2009a). The critical role of inflammatory cells in cancers that cannot be linked to a pre-existing inflammatory condition has been recognized only recently (Mantovani et al., 2008). Kim et al. (2009), and other studies (Mantovani et al., 2008; Coussens et al., 2002), suggested to include cancer-related inflammation (CRI) as additional hallmark. Recently, there has been a renaissance of the inflammation-cancer connection. Therefore, inflammatory

responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis (Grivennikov et al., 2010).

Many dietary bioactive compounds have been identified as potential anti-inflammatory agents, but their mechanisms of action have not been fully investigated. The anti-inflammatory activity of different single flavonoids (luteolin, tangeretin, sulforaphane) has been evaluated in Raw 264.7 cells-macrophages *in vitro*, in order to elucidate their action mechanisms against cancer (Cerella et a., 2010; Pan et al., 2009; Xiao, 2009). In view of the fact that flavones content of vegetal products is highly variable, luteolin is an compound that have shown high anti-inflammatory properties (Harris et al., 2006; Park et al., 2011) and can be isolated from thyme and sage leaves, celery and peppers (http://www.nal.usda.gov/fnic/foodcomp/Data/Other/EB03_VegFlav.pdf).

Another flavonoid with potent biological activities, such as anti-cancer (Chen et al., 2007b; Meiyanto et al., 2012) and anti-inflammatory (Murakami et al., 2012), is tangeretin (polymethoxyflavone (PMF), which is present almost exclusively in citrus plants (Li et al., 2009). Tangeretin is metabolized in rat and human liver microsomes by an *O*-demethylation reaction involving cytochrome P450 (Canivenc-Lavier et al., 1993). Glucosinolates, others nutraceutical compounds, are abundant in various cruciferous vegetables including cabbage, Brussel sprout, broccoli, cauliflower, horseradish, mustard, watercress, turnips, radish, rutabaga and kohlrabi. In plants, sulforaphane and others isothiocyanates (ITCs) are synthesized by myrosinase enzyme and stored in cells as a glucosinolate (gluocoraphanin in the case of sulforaphane). Sulforaphane is a plant-derived aliphatic ITC, whose cancer chemopreventive activity was recognized after 1992, when it was found to be the principal ingredient of broccoli extracts exhibiting potent induction of phase 2 detoxification enzymes (Zhang et al., 1992). Furthermore, cruciferous

vegetables intake have demonstrated a protective anti-tumor action against of colon, rectum and mammary cancer (Whitmore, 2000). More recent observations suggest that it may also induce membrane transporters to remove carcinogens from cells, inhibit inflammation, downregulate ornithine decarboxylase, and eliminate *Helicobacter pylori*, which is linked to stomach cancer (Zhang, 2004). Identifying potential cancer preventive dietary components, elucidating their molecular mechanisms, and investigating possible synergistic interactions, will be the long-term goal of developing diet-based strategies for cancer prevention. .

The aim of this work was to evaluate the individual and combined effects of several dietary food components (thymol (THY), polymethoxyflavones (PMFs), curcumin derivates (CURC), luteolin (LUT), sulforaphane (SFN), and docosa-4,7,10,13,16,19-hexaenoic acid (DHA) against lipopolysaccharide (LPS)-induced acute inflammation, with emphasis on the role of anti-inflammatory (heme oxygenase-1 (HO-1)) and pro-inflammatory enzymes (inducible nitric oxide (iNOS) and cyclooxygenase2 (COX-2)).

5.3 Materials and Methods (Guo et al., 2012)

Cell culture

RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in RPMI-1640 media supplemented with 10% heat-inactivated FBS (Medistech, Herndon, VA), 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO) at 37 °C with 5% CO_2 and 95% air. Dimethyl sulfoxide (DMSO) was used as the vehicle to deliver THY (>98%), DHA (>98%), PMFs (>98%), CURC (>98%), LUT (>98%), SFN (>98%), to the culture media, and the final concentration of DMSO in all experiments was 0.1% (v/v) in cell culture media.

Cell viability assay

The cell viability was determined as previously described (Xiao et al., 2009). Briefly, RAW 264.7 cells (50000 cells/well) were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of test compounds in 200 μL of serum complete media. After 24 h treatments, cells were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Media were replaced by 100 μL of fresh media containing 0.1 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO). After 2 h of incubation at 37 °C with 5% CO_2 and 95% air, MTT-containing media were removed, and the reduced formazan dye was solubilized by adding 100 μL of DMSO to each well. After gentle mixing, the absorbance was monitored at 570 nm using a plate reader (Elx800TM absorbance microplate reader, BioTek Instrument, Winooski, VT).

NO assay

The nitrite concentration in the culture media was measured as an indicator of NO production by the Griess reaction. The culture media were mixed with an equal volume of Griess reagent A and B (A, 1% sulfanilamide in 5% phosphoric acid; and B, 0.1% naphthylethylenediamine dihydrochloride in water). To stimulate inflammation in culture cells lipopolysaccharide (LPS) from *Escherichia Coli* O55:B5 was used. The absorbance of the mixture was measured at 540 nm by a plate reader, and concentrations of nitrite were calculated according to a standard curve constructed with sodium nitrite as a standard.

Analyses of synergy

The analyses were based on the isobologram method as described previously (Xiao et al., 2008b). It was assumed that the dose−response model follows $\log[E/(1 - E)] = \alpha(\log d - \log D_m)$, which is a linear regression model with the response $\log[E/(1 - E)]$ and the regressor log (d). This model is used for compound 1, compound 2, and the combinations of the two compounds with a fixed ratio of their doses. E is fraction of NO production, d is the dose applied, D_m is the median effective dose of a compound, and α is a slope parameter. On the basis of this regression model, median effect plots were constructed using data from the NO assay. Suppose that the combination (d_1, d_2) elicits the same effect x as compound 1 alone at dose level $D_{x,1}$ and compound 2 alone at dose $D_{x,2}$, then, the interaction index = $d_1/D_{x,1} + d_2/D_{x,2}$ ($D_{x,1}$ and $D_{x,2}$ were calculated from median effect models). The interaction index was used to determine additivity, synergy, or antagonism of the combination at dose (d_1, d_2) depending on interaction index = 1, <1, or >1, respectively. The δ method (http://en.wikipedia.org/wiki/Delta_ method) was used to calculate the variance of the interaction index, which is given by var. (interaction index) = var. $(D_{x,1})(d_1^2/D_{x,1}^4)$ + var.$(D_{x,2})(d_2^2/D_{x,2}^4)$. Data were analyzed by R program (http://www.rproject.org/).

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells (5×10^6 cells/well) were seeded in 6-well. After 24 h, cells were treated with 1 μg/mL LPS alone or with serial concentrations of test compounds in 2 mL of serum complete media. After another 24 h of incubation, the culture media were collected and analyzed for IL-1, IL-6 and PGE2 levels by ELISA kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Preparation of whole cell lysate

RAW 264.7 cells were washed with ice-cold PBS and collected with cell scrapers from culture plates. The cells were combined with floating cells, if any, and incubated on ice in lysis buffer containing RIPA with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Boston Bioproducts, Boston, MA) for 20 min on ice. Cell suspensions were then subjected to sonication (5 s, seven times). After further incubation for 20 min on ice, supernatants were collected by centrifugation at 14000 rpm (23447 x g) for 20 min. Protein concentrations were determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL), following the manufacturer's instructions.

Immunoblot analysis

For immunoblot analysis, equal amount of proteins (50 μg) were resolved over 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, proteins of interest were probed using different antibodies at the manufacturer's recommended concentrations and then visualized using enhanced chemiluminescence (Boston Bioproducts, Ashland, MA). Antibodies for iNOS, COX-2, HO-1 were purchased from Cell Signaling Technology (Beverly, MA). Anti-β actin antibody was from Sigma-Aldrich (St. Louis, MO).

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) analysis

Total Ribonucleic acid (RNA) of RAW 264.7 cells was isolated by RNeasy Plus Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA concentrations were determined using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). From each sample, 0.16 mg of total RNA was converted to single-stranded cDNA, which

was then amplified by Brilliant II SYBR Green QRT-PCR Master Mix Kit, 1-Step (Agilent Technologies, Santa Clara, CA) to detect quantitatively the gene expression of iNOS, COX-2, HO-1, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (as an internal standard) using Mx3000P QPCR System (Stratagene, La Jolla, CA). The primer pairs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The primer pairs were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and the sequences are listed in Table 14.

A minimum of three independent experiments was carried out, and each experiment had triplicate samples for each treatment. The copy number of each transcript was calculated with respect to the GADPH copy number, using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

Table 14. Oligonucleotide primers used for qRT-PCR (from Guo et al., 2012).

gene	sequence
iNO	F: 5′-TCC TAC ACC ACA CCA AAC-3′
	R: 5′-CTC CAA TCT CTG CCT ATC C-3′
COX-2	F: 5′-CCT CTG CGA TGC TCT TCC-3′
	R: 5′-TCA CAC TTA TAC TGG TCA AAT CC-3′
HO-1	F: 5′-AAG AGG CTA AGA CCG CCT TC-3′
	R: 5′-GTC GTC GTC AGT CAA CAT GG-3′
IL-1	F: 5′-GAG TGT GGA TCC CAA GCA AT-3′
	R: 5′-CTC AGT GCA GGC TAT GGA CCA-3′
GAPDH	F: 5′-TCA ACG GCA CAG TCA AGG-3′
	R: 5′-ACT CCA CGA CAT ACT CAG C-3′

Statistical analysis

All data were presented as the mean ± SD. Student's t test was used to test the mean difference between two groups, using PASW Statistics, 17. An analysis of variance (ANOVA) model was used for the comparison of differences among more than two groups. Overall differences between means were tested according to Tukey's test, performed at 95% confidence level and considered to be significant when $P < 0.05$ (PASW Statistics, 17).

5.4 Results and Discussion

Evaluation of anti-inflammatory effects of thymol on nitrite concentration (NO assay) in RAW 264.7 cells-macrophages

Several studies are focusing on thyme antioxidant and anticancer properties, because it is rich of flavonoids with healthy biological effects (Boros et al., 2010). In this experimental research, the anti-inflammatory effect of thymol was evaluated at different concentration (4-20 μM; 10-50 μM; 20-100 μM) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages cells. However, none concentration range had significant effects. Some interesting results were only detected at a very high concentration (400 μM), but it affects cell physiology as it leads to cellular death. For that reasons, it was not investigated how thymol treatment could affect protein expression levels.

Evaluation of synergistic anti-inflammatory effects of polymethoxyflavones (PMFs) and docosa-4,7,10,13,16,19-hexaenoic acid (DHA) on nitrite concentration (NO assay) in RAW 264.7 cells-macrophages.

It was evaluated the anti-inflammatory effect of several PMFs in combination with DHA:

- Tangeretin (5,6,7,8,4'-pentamethoxylflavone) (6-30 µM);
- 4'-demethyltangeretin (4'-hydroxy-5,6,7,8-tetramethoxylflavone) (2-10 µM);
- 5-demethyltangeretin (4'-hydroxy-6,7,8,4'-tetramethoxylflavone) (0.25-2 µM);
- 5,4-didemethyltangeretin (5,4'-dihydroxy-6,7,8-trimethoxylflavone) (2-10 µM);
- Nobiletin (5,6,7,8,3',4'-hexamethoxylflavone (10-50 µM);
- 3'-demethylnobiletin (4'-hydroxy-5,6,7,8,3'-pentamethoxylflavone) (10-50 µM)
- 4'-demethylnobiletin (3'-hydroxy-5,6,7,8,4'-pentamethoxylflavone) (2-10 µM)
- 3',4'-didemethylnobiletin (3',4'-dihydroxy-5,6,7,8,-tetramethoxylflavone) (2-10 µM)
- 5'-demethylnobiletin (5'-hydroxy-6,7,8,3',4'-pentamethoxylflavone) (2-10 µM)
- 5,3'-didemethylnobiletin (5,3'-dihydroxy-6,7,8,4'-tetramethoxylflavone) (2-10 µM)
- 5,4'-didemethylnobiletin (5,4'-dihydroxy-6,7,8,3'-tetramethoxylflavone) (1-5 µM)
- 5,3',4'-tridemethylnobiletin (5,3',4'-trihydroxy-6,7,8-trimethoxylflavone) (3-15 µM)

Every single PMF in combination with DHA (10-50 µM) showed not significant synergic anti-inflammatory effects in Raw 264.7 cells-macrophages-LPS stimulated. For that reason, it was not further investigated how PMFs+DHA combination could affect protein expression levels.

Evaluation of synergistic anti-inflammatory effects of luteolin and docosa-4,7,10,13,16,19-hexaenoic acid (DHA) on nitrite concentration (NO assay) in RAW 264.7 cells-macrophages

Luteolin (3-15 µM) in combination with DHA (10-50 µM), did not report synergistic anti-inflammatory effects in Raw 264.7 cells-macrophages-LPS stimulated. The results showed that combined luteolin and DHA treatments did not produced much stronger effects on the production of nitric oxide (NO) than the single compounds at the highest concentrations. Overall, the luteolin and DHA combination was not useful to figure out protein expression levels as well.

Evaluation of synergistic anti-inflammatory effects of curcumin derivates (CURC) and docosa-4,7,10,13,16,19-hexaenoic acid (DHA) on nitrite concentration (NO assay) in RAW 264.7 cells-macrophages

CURC (C1, 3-15 µM; C2, 4-20 µM; C3, 4-20 µM; C4, 2-10 µM; C5, 2-10 µM; C6, 4-20 µM; C7, 2-10 µM) in combination with DHA (10-50 µM) did not exhibit synergistic anti-inflammatory effects in Raw 264.7 cells-macrophages-LPS stimulated. The results showed that combined curcumin derivatives and DHA treatments did not produced much stronger effects on the production of nitric oxide (NO) than the single compounds at the highest concentrations. Overall, the curcumin derivatives and DHA combination was not useful to elucidate protein behavior as well.

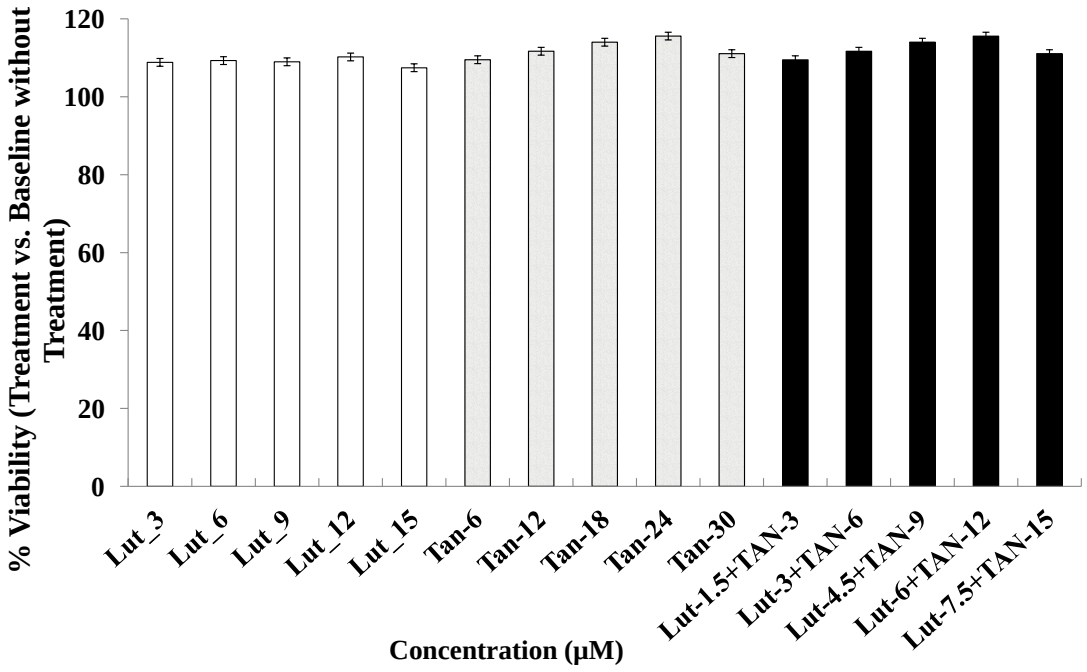
Examples of synergistic anti-inflammatory effects in LPS-stimulated RAW 264.7 cells

LUT+TAN and LUT+SFN in combination produced synergistic effects in inhibiting LPS-induced inflammation in macrophage cells. To establish nontoxic dose ranges for LUT, TAN and SFN in RAW 264.7 cells, the effects of all of these compounds on the cell viability were determined using the MTT assay (Xiao et al., 2009). The effects of both LUT+TAN and LUT+SFN combinations on the cell viability, were further tested. The results showed (Figure 7A and 7B) that the combined treatments LUT+TAN (at about 1:2 ratio, up to 7.5 μM Lut + 15 μM TAN) and LUT+SFN (at about 12:1 ratio, up to 7.5 μM Lut + 0.6 μM SFN) did not cause a significant decrease on the viability of RAW 264.7 cells. Stimulation of murine RAW 264.7 cells macrophages with a bacterial toxin, lipopolysaccharide (LPS) for 24 h, resulted in NO generation and then nitrite (NO_2^-) accumulation in the media. As shown in Figure 8A and 9A, individual treatment of LUT, TAN and SFN, at a concentration range of 3-15 μM, 6-30 μM, and 0.24-1.20 μM, respectively, caused a dose-dependent inhibition on LPS-induced NO production. As shown in Figure 8A, LUT caused a dose-dependent inhibition on LPS-induced NO production by 12, 33, 48, 68 and 77% at 3, 6, 9, 12 and 15Mm, respectively. Similarly, TAN treatment also resulted in a dose-dependent inhibition on NO production by 15, 21, 22, 30 and 44% at 6, 12, 18, 24 and 30 μM, respectively. As shown in Figure 8A, combinations of half dose of LUT with half dose of TAN resulted in stronger inhibition on NO production than the individual treatments with LUT or TAN at full doses. Furthermore, co-treatment with LUT and TAN synergistically inhibited NO production. For example, a combination of 1.5 μM of LUT with 3 μM of TAN caused a 19% inhibition on NO production, while 3 μM of LUT alone and 6 μM of TAN alone led to only 12 and 15% inhibition, respectively. A combination of 3 μM of LUT with 6 μM of TAN caused a 42% inhibition on

NO production, while 6 μM of LUT alone and 12 μM of TAN alone led to only 33 and 21% inhibition, respectively (Figure 8A). As shown in Figure 9A, combinations of half dose of LUT with half dose of SFN resulted in stronger inhibition on NO production than the individual treatments with LUT or SFN at full doses. Furthermore, co-treatment with LUT and SFN synergistically inhibited NO production as well. For example, a combination of 1.5 μM of LUT with 0.12 μM of SFN caused a 29% inhibition on NO production, while 3 μM of LUT alone and 0.24 μM of SFN alone led to only 11 and 22% inhibition, respectively; a combination of 4.5 μM of LUT with 0.36 μM of SFN caused a 62% inhibition on NO production, while 9 μM of LUT alone and 0.72 μM of SFN alone led to only 32 and 30% inhibition, respectively. The mode of interaction between both dose pairs in inhibiting NO production, was determined by using isobologram analysis as described previously (Xiao et al., 2008b). In Figures 8B and 9B, the median effect plot shows that the linear regression model used in the isobologram analysis well fitted the dose-response relationship of LUT, TAN, LUT+TAN and LUT, SFN, LUT+SFN within the concentration ranges exploited herein. On the basis of the median effect plot, the interaction indexes of each combinations dose pair were calculated as described in the Materials and Methods. The interaction index was used to determine additivity, synergy, or antagonism of the combination at different doses depending on interaction index =1, <1, or >1, respectively. As shown in Figures 8C and 9C, all five dose pairs tested, for each combination treatment, resulted in an interaction index lower than 0.8, suggesting a synergistic interaction between LUT+TAN and LUT+SFN in terms of inhibiting NO production in LPS-stimulated macrophages.

Figure 7: (A) Cytotoxicity profile of LUT, TAN, and their combinations in RAW 264.7 macrophage cells. (B) Cytotoxicity profile of LUT, SFN, and their combinations in RAW 264.7 macrophage cells. The viability of control cells was set as the reference with a value of 100%. Results are presented as means ± SDs from six replicates (n = 6). N.s. not significant difference (*P* < 0.01).

(A)



(B)

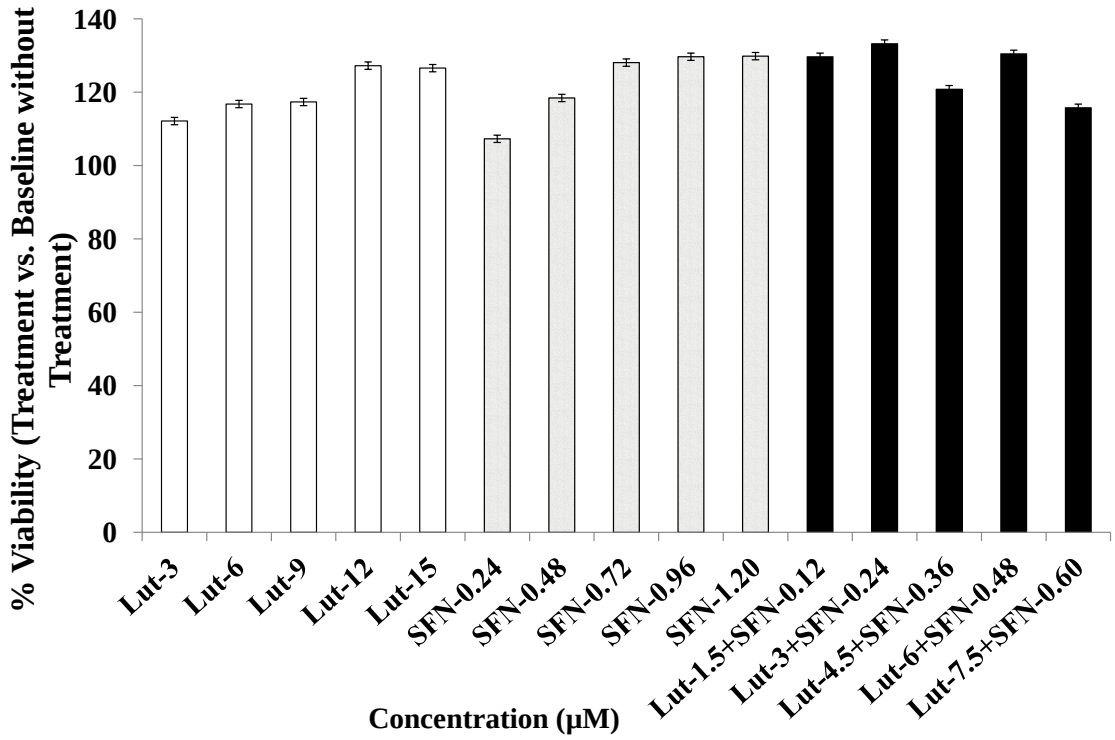
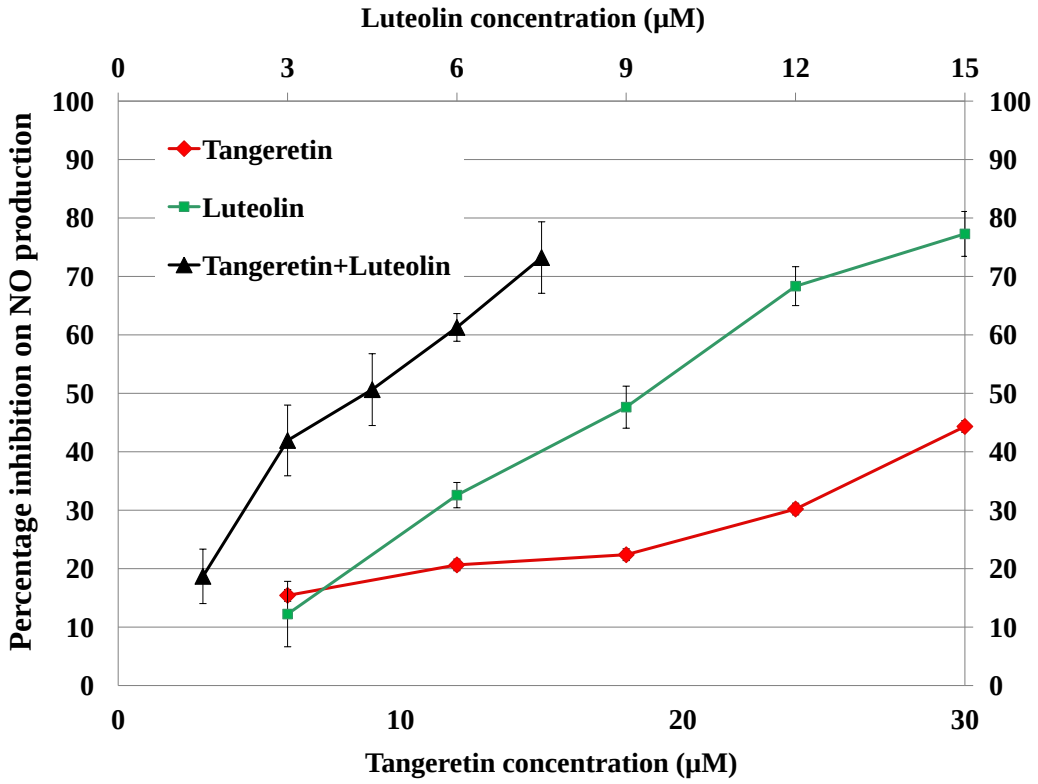
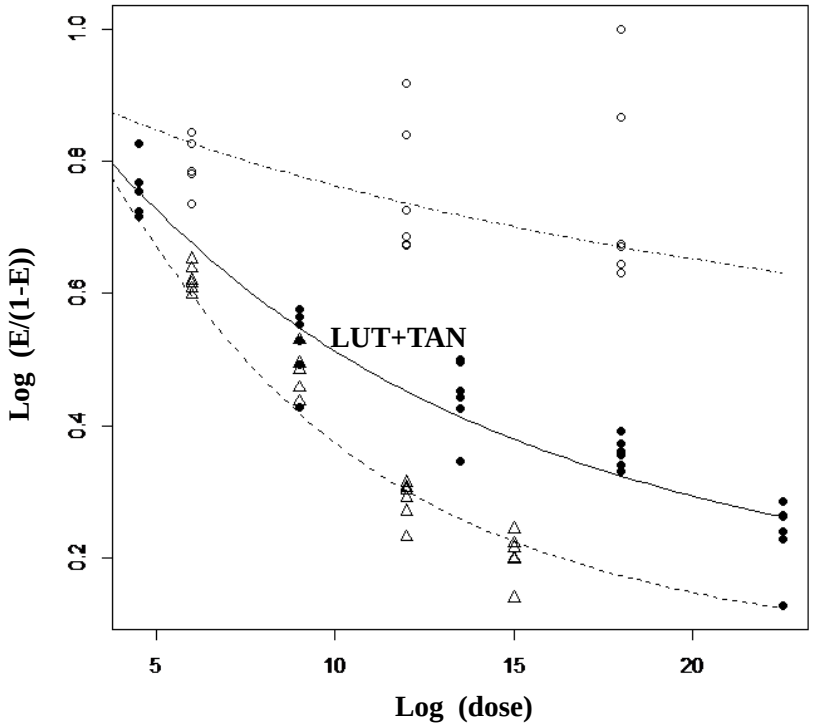

% Viability (Treatment vs. Baseline without Treatment)
140
120
100
80
60
40
20
0
Lut-3
Lut-6
Lut-9
Lut-12
Lut-15
SFN-0.24
SFN-0.48
SFN-0.72
SFN-0.96
SFN-1.20
Lut-1.5+SFN-0.12
Lut-3+SFN-0.24
Lut-4.5+SFN-0.36
Lut-6+SFN-0.48
Lut-7.5+SFN-0.60
Concentration (µM)

Figure 8: (A) Percentage of inhibition on NO production by LUT, TAN, and their combinations in LPS-stimulated RAW 264.7 cells. (B) Median effect plot of LUT, TAN, and their combination on inhibition of NO production in LPS-stimulated RAW 264.7 cells. (C) Interaction index plot for the combination effects of LUT and TAN on NO production in LPS-stimulated RAW 264.7 cells. Results are presented as means ± SDs from six replicates. All treatments showed statistical significance as compared to the positive control in A. ($P < 0.01$, n =6).

(A)



(B)



(C)

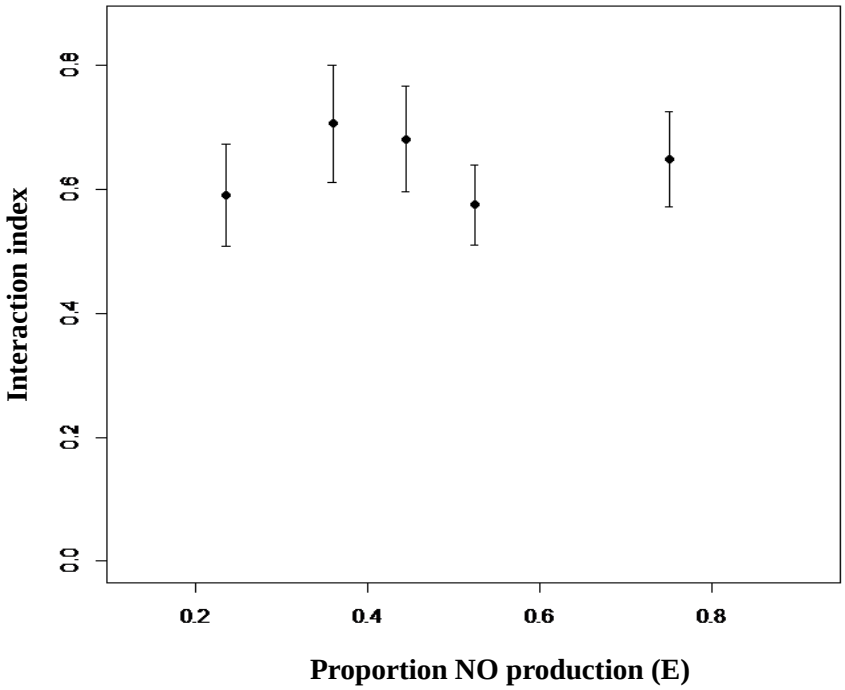
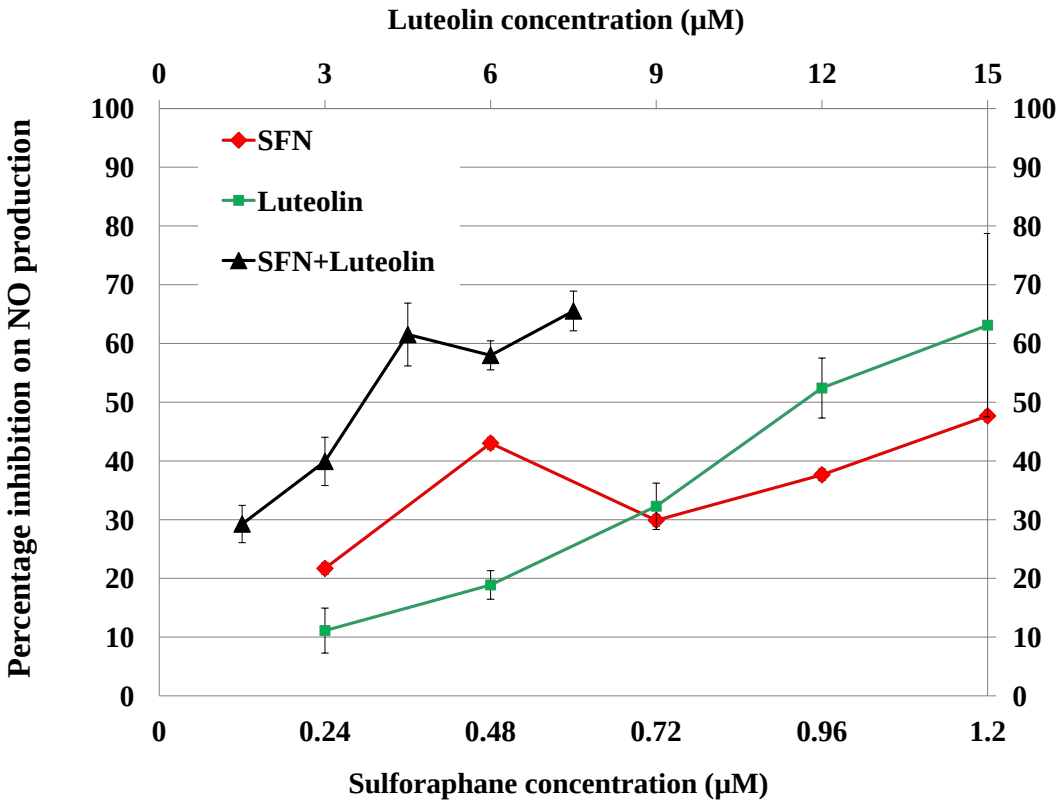
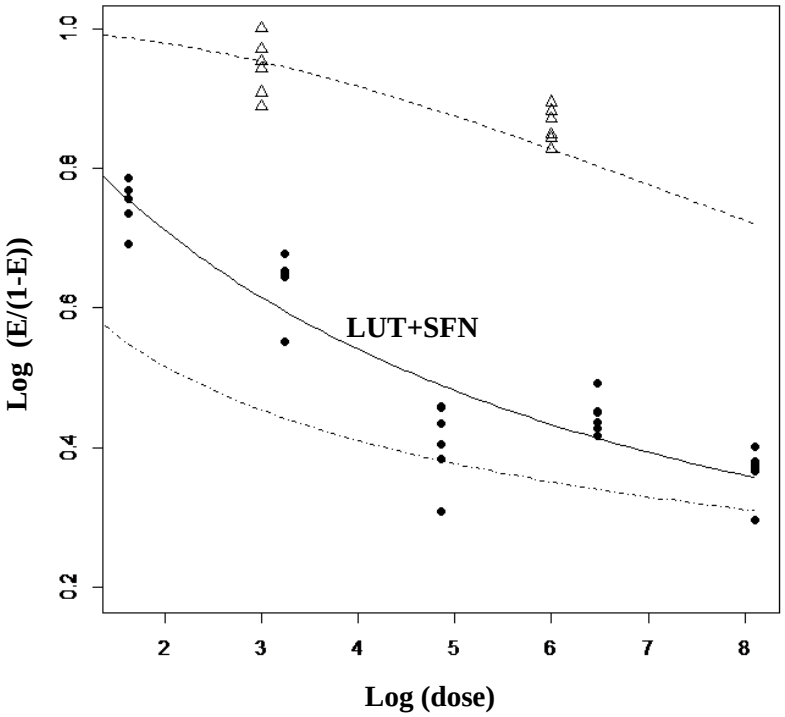

Interaction index
0.0
0.2
0.4
0.6
0.8
0.2
0.4
0.6
0.8
Proportion NO production (E)

Figure 9: (A) Percentage of inhibition on NO production by LUT, SFN, and their combinations in LPS-stimulated RAW 264.7 cells. (B) Median effect plot of LUT, SFN, and their combination on inhibition of NO production in LPS-stimulated RAW 264.7 cells. (C) Interaction index plot for the combination effects of LUT and SFN on NO production in LPS-stimulated RAW 264.7 cells. Results are presented as means ± SDs from six replicates. All treatments showed statistical significance as compared to the positive control in A. ($P < 0.01$, n =6).

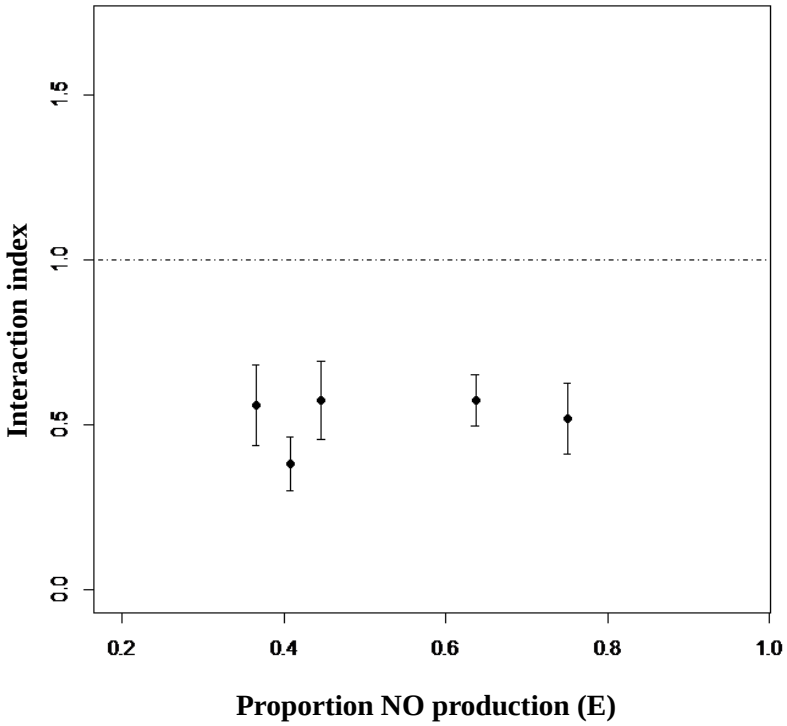
(A)



(B)



(C)


Interaction index
0.0
0.5
1.0
1.5
0.2
0.4
0.6
0.8
1.0
Proportion NO production (E)

Combination of LUT-TAN on proteins level (iNOS, COX-2, HO-1)

Since NO production was synergistically suppressed by combination of LUT and TAN in LPS-stimulated RAW 264.7 cells, the effects of this combination on the level of iNOS and COX-2 (pro-inflammatory proteins), and HO-1 (anti-inflammatory protein) were then examined. To elucidate the molecular mechanism of interaction between LUT+TAN, their effects on these important pro-inflammatory proteins were investigated. To test the effects, 7.5μM of Lut + 15 μM Tan was compared with LUT and TAN alone; the ratio of LUT:TAN was 1:2, which was the same as that in Figures 8A. LPS-stimulated RAW 264.7 cells were treated with each individual (15 μM of Lut, 30 μM Tan) and combinatorial (half and full dose) compounds for 24 h, and then the protein levels of each protein in RAW 264.7 cells were determined by Immunoblot analysis. The results demonstrated that 15 μM of LUT and 30 μM of TAN significantly decreased iNOS protein levels by 50 and 32%, respectively, as compared to the LPS-treated positive control cells (Figure 10). However, the combination of LUT + TAN at full doses produced a 77% decrease on iNOS protein level, which was stronger than the effects produced by LUT and TAN alone. Treatment effects were also determined on protein levels of COX-2, an important pro-inflammatory protein. The results showed that 15 μM of LUT + 30 μM of TAN (full dose), and 7.5 μM of LUT + 15 μM of TAN (half dose) caused significant decrease on protein levels of COX-2 by 99. 91%, and 99.99%, respectively, as compared to the LPS-treated positive control cells (Figure 10). Furthermore, Immunoblot analysis showed that combined LUT and TAN pretreatments synergistically decreased LPS-induced COX-2 and iNOS protein expression levels.

Using the qRT-PCR technique, the effects of LUT, TAN, and their combination on mRNA levels of iNOS and COX-2, were determined after 24 h treatments. LUT, TAN, or their combination decreased mRNA levels of COX-2, suggesting that they may decrease the protein level of COX-2 by

other mechanisms, such as modulating translation and/or degradation of COX-2 protein in LPS-stimulated macrophage cells. Interestingly, LUT, TAN and their combination caused a strong decrease on mRNA levels of iNOS and COX-2. This result suggested that LUT and TAN had similar mechanisms to downregulate iNOS and COX-2 proteins.

As shown in Figures 11A and 11B, the LPS treatments significantly increased the messenger Ribonucleic acid (mRNA) levels of both iNOS and COX-2, which is consistent with the increased protein expression levels of the two proteins after LPS treatment. Single treatment of LUT and TAN did decrease mRNA levels of iNOS or COX-2, but both combinations, especially full dose combination, had a much stronger effect (Figures 11A and 11B), similar to iNOS and COX-2 protein expression proteins as shown in Figure 10.

Anti-inflammatory effects of LUT alone have been studied both *in vitro* and *in vivo*, demonstrating that luteolin dose-dependently inhibited the expression and the production of inflammatory genes and mediators (Chen et al., 2007a; Xagorari et al., 2001). Moreover, anti-inflammatory effects of TAN alone have been studied *in vitro* demonstrating that tangeretin suppresses IL-1β-induced COX-2 expression in cancer cells (Chen et al., 2007b).

Luteolin and chrysin differentially showed anti-inflammatory effects affecting inflammation-associated Cox-2 expression and PGE-2 formation in RAW 264.7 (Harris et al., 2006).

Co-treatment with LUT and chicoric acid synergistically reduced cellular concentrations of nitric oxide (NO) and prostaglandin E_2 (PGE-2) and also inhibited expression of iNOS and COX-2 (Park et al., 2011). Targeting COX-2 expression by natural compounds may therefore represent a promising alternative strategy for cancer chemoprevention and therapy (Cerella et al., 2010). Many studies have demonstrated that pro-inflammatory cytochines such as IL-1, IL-6, and PGE-2, play important roles in the progression of various diseases including tumors.

The effects of LUT, TAN and their combination on the production of all these metabolites in LPS-stimulated RAW 264.7 cells, were determined by using ELISA assay. The results showed that 15 μM of LUT + 30 μM of TAN (full dose), and 7.5 μM of LUT + 15 μM of TAN (half dose) caused significant decrease on production of IL-1, IL-6 and PGE-2, especially full dose combination, as compared to the LPS-treated positive control cells (Figure 12A-C). Both combination treatment (half and especially full dose) exhibited the highest decrease of cytokines production as compared to LPS-treated positive control cells.

It is suggested that high levels of proinflammatory cytokines can stimulate tumor growth and progression (Lin et al., 2007). The results obtained showed that LUT+TAN (especially full dose) did cause a strong inhibition on IL-1, IL-6 and PGE-2 levels.

One of the major biological effects of LUT and TAN is induction of phase II enzymes, which in turn contributes to cancer preventive activity of each compound. HO-1 is an important phase II antioxidant enzyme, as it plays a role in regulating inflammation response and in protecting against the cytotoxicity of oxidative stress and apoptotic cell death (Paine et al., 2010).

As shown in Figure 10, protein expression levels of HO-1 in LPS-stimulated RAW 264.7 cells, which resulted in a noticeable increase, especially in full dose combination. Although each single compound did significantly increase the HO-1 protein level, the combination of LUT+TAN at full doses increased the HO-1 protein level by 6.15-fold as compared to the control (Figure 10). Furthermore, the effects of LUT, TAN and their combination on the protein and mRNA levels of HO-1 in LPS-stimulated macrophage cells, were evaluated. As shown in Figure 11C, the results demonstrated that half and full dose combination of LUT+TAN and LUT+SFN increased the HO-1 protein level with respect to the control cells. The present results proved that single and combination treatment can increase the HO-1 protein level in RAW 264.7

cells. Therefore, consistent with these results on HO-1 protein levels, LUT, TAN, and their combination increased also the mRNA level of HO-1.

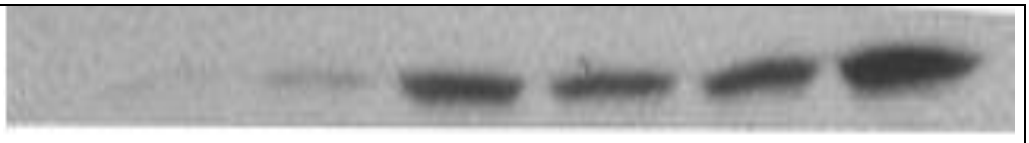
The results here obtained are then in agreement with Paine et al. (2010), who demonstrated that the expression of HO-1 is crucial in inhibiting LPS-induced pro-inflammatory responses in RAW 264.7 cells.

Figure 10: Inhibitory effects of LUT, TAN, and their combination on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 cells. The numbers underneath of the blots represent band intensity (normalized to β-actin, means of three independent experiments) measured by Image J software. β-Actin served as an equal loading control. * indicates a statistical significance (*P* < 0.05, n = 3).

	CTL	LPS	LUT	TAN	HALF	FULL
LPS	-	+	+	+	+	+
LUT (μM)	-	-	15	-	7.5	15
TAN (μM)	-	-	-	30	15	30

iNOS						
		1.00	**0.50 ***	**0.68 ***	**0.75 ***	**0.33 ***

COX-2						
		1.00	**0.89 ***	**0.14 ***	**0.09 ***	**0.01 ***

HO-1						
	1.00	**1.15**	**3.63 ***	**3.03 ***	**3.49 ***	**6.15 ***

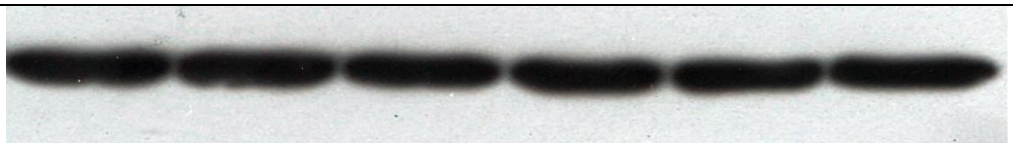
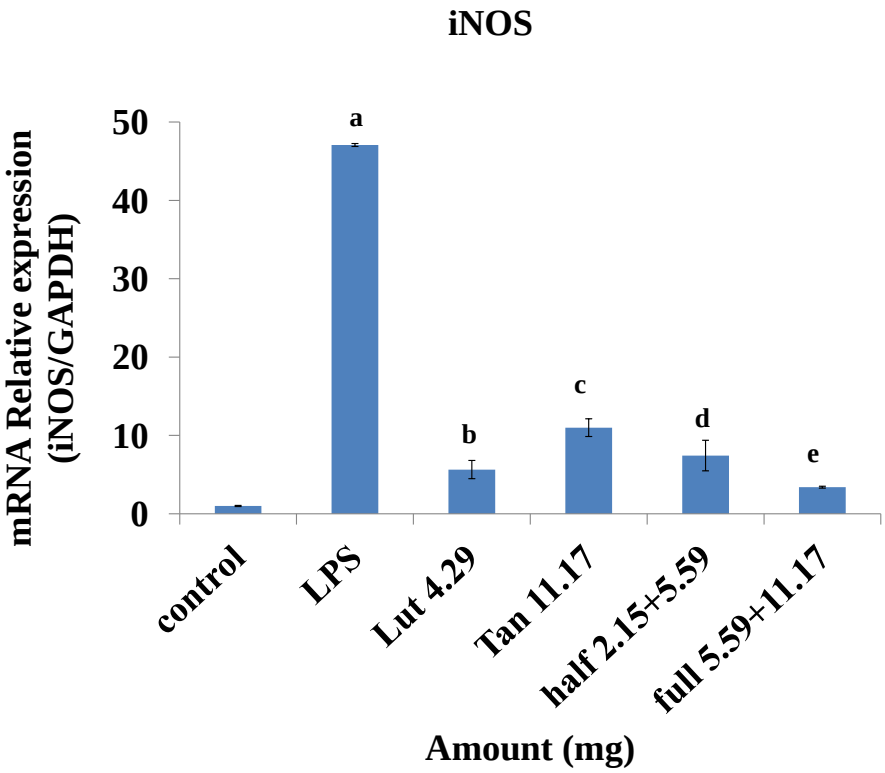
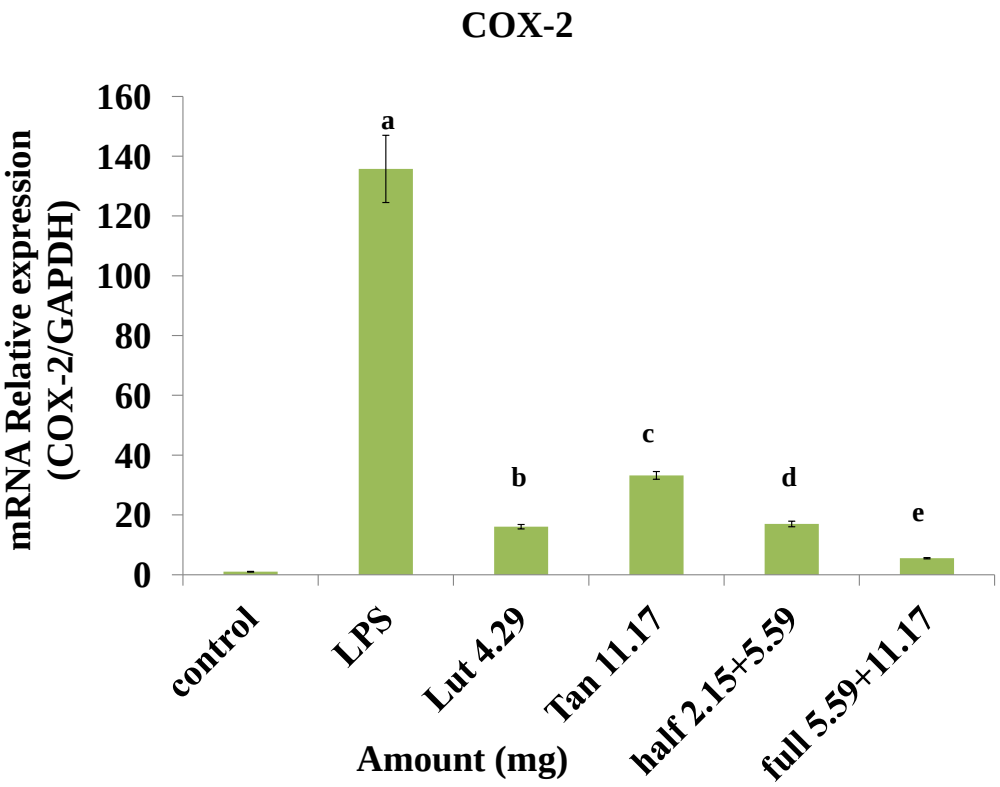
β-actin	

Figure 11: Effects of LUT, TAN, and their combination on mRNA levels of iNOS (A), COX-2 (B) and HO-1 (C) in LPS-stimulated RAW 264.7 cells. The cells were treated with LPS or LPS plus LUT, TAN, and their combination at concentrations indicated in the figure. Each value represents the mean ± SD of three independent experiments. Different annotations indicate statistical significance ($P < 0.01$, n = 3) by ANOVA.

(A)



(B)


COX-2
mRNA Relative expression (COX-2/GAPDH)
160
140
120
100
80
60
40
20
0
a
b
c
d
e
control
LPS
Lut 4.29
Tan 11.17
half 2.15+5.59
full 5.59+11.17
Amount (mg)

(C)

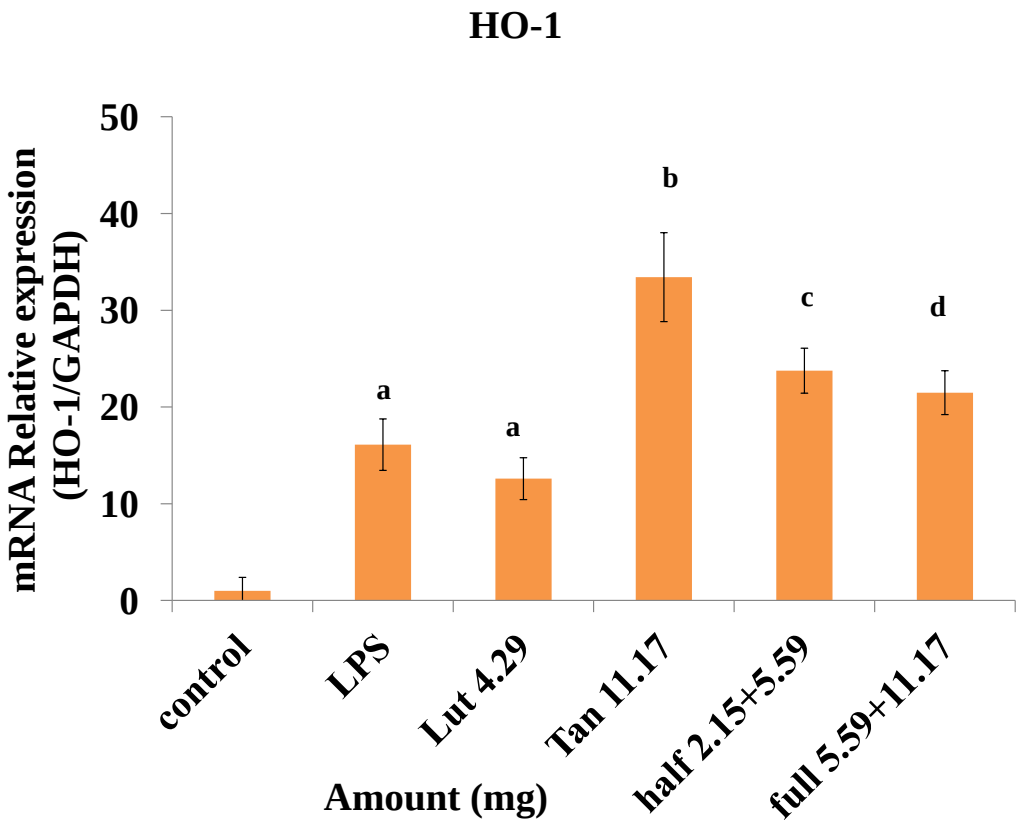
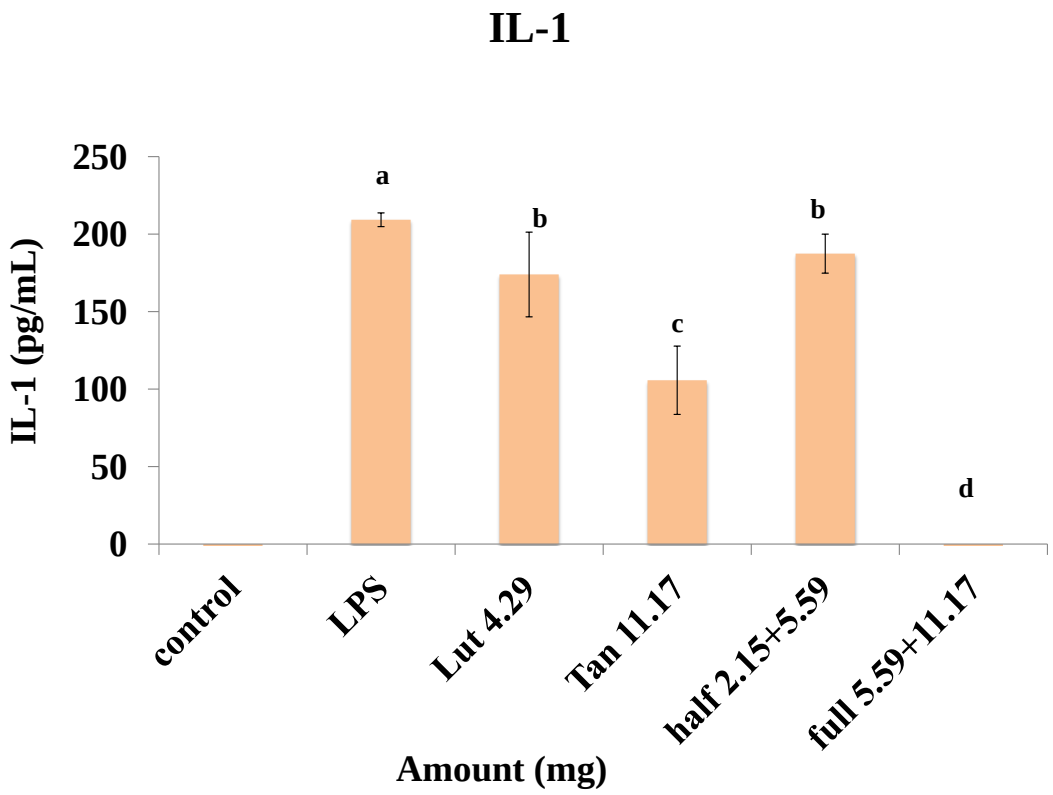
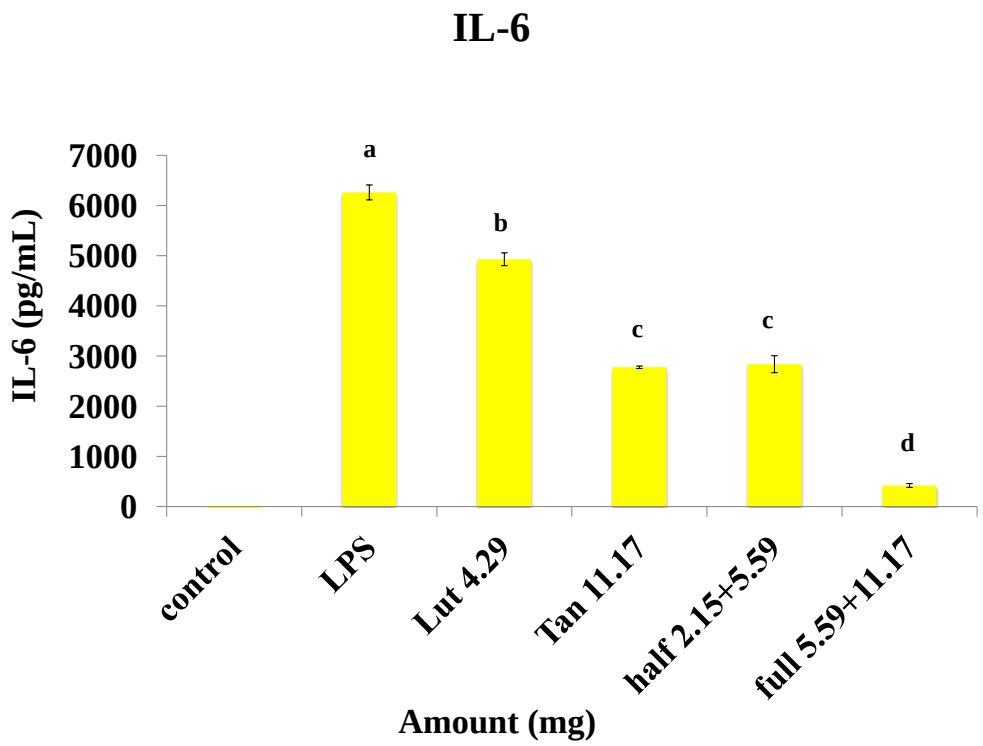

HO-1
mRNA Relative expression (HO-1/GAPDH)
50
40
30
20
10
0
a
a
b
c
d
control
LPS
Lut 4.29
Tan 11.17
half 2.15+5.59
full 5.59+11.17
Amount (mg)

Figure 12: Effects of LUT, TAN and their combination on protein expression levels of IL-1 (A) IL-6 (B) PGE2 (C) in LPS-stimulated RAW 264.7 cells Each value represents the mean ± SD of three independent experiments. Different annotations indicate statistical significance (*P* < 0.01, n = 3) by ANOVA.

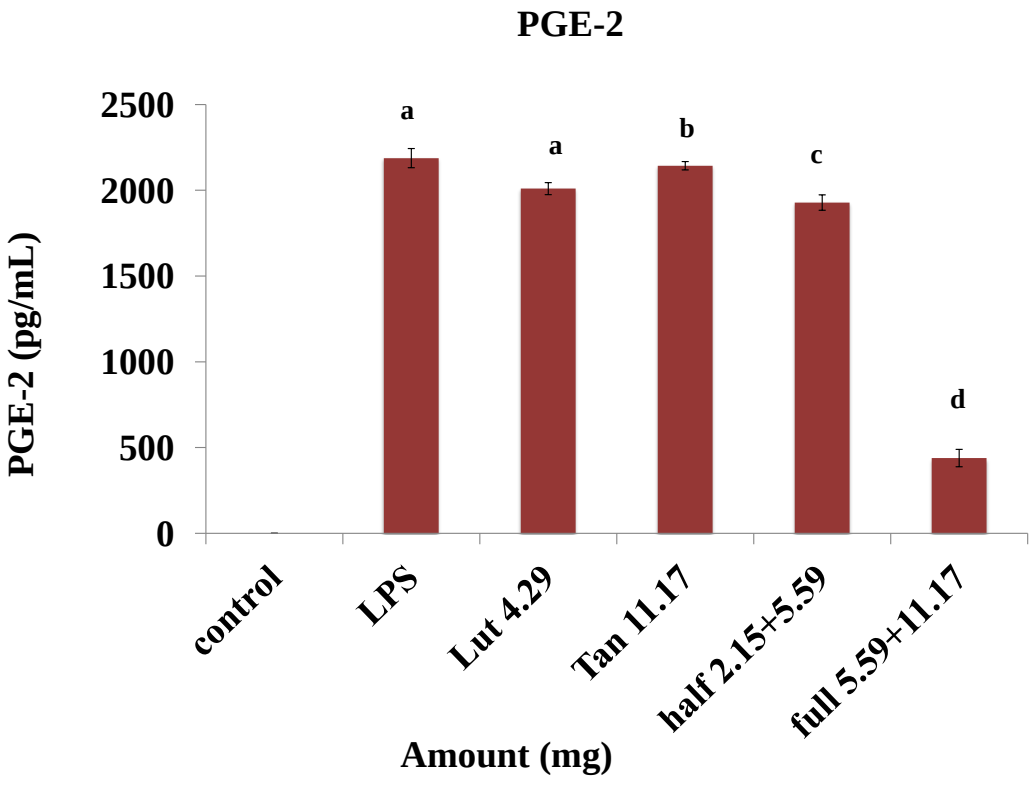
(A)



(B)


IL-6
IL-6 (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
a
b
c
c
d
control
LPS
Lut 4.29
Tan 11.17
half 2.15+5.59
full 5.59+11.17
Amount (mg)

(C)


PGE-2
PGE-2 (pg/mL)
2500
2000
1500
1000
500
0
a
a
b
c
d
control
LPS
Lut 4.29
Tan 11.17
half 2.15+5.59
full 5.59+11.17
Amount (mg)

Combination of LUT-SFN and protein levels (iNOS, COX-2 and HO-1)

Since NO production was synergistically suppressed by combination of LUT and Tan in LPS-stimulated RAW 264.7 cells, the effects of this combination on the level of iNOS and COX-2 (pro-inflammatory proteins), and HO-1 (anti-inflammatory protein) were then evaluated. To elucidate the molecular mechanism of interaction between LUT+SFN, their effects on these important pro-inflammatory proteins were investigated. To test these effects, LPS-stimulated RAW 264.7 cells were treated with each individual (15 μM of Lut, 1.2 μM SFN) and combinatorial (half and full dose) compounds for 24 h, and then the protein levels of each protein in RAW 264.7 cells were determined by Immunoblot analysis. The results demonstrated that single treatment of LUT (15 μM) and SFN (1.2 μM) significantly decreased iNOS protein levels by 67 and 82%, respectively, as compared to the LPS-treated positive control cells (Figure 13). However, the combination of LUT and TAN at full doses produced a 88% decrease on iNOS protein level, which was stronger than the effects produced by LUT and TAN alone. Overall, LUT and SFN combination gave similar results to those obtained with the LUT and TAN combination, except for COX-2 protein expression. In fact, each treatment on protein expression of COX-2 showed controversial increased effects, as compared to the LPS-treated positive control cells (Figure 13). Furthermore, Immunoblot analysis revealed that combined LUT and SFN pretreatments synergistically decreased LPS-induced iNOS protein expression levels and increased HO-1 protein expression. The effects of LUT, SFN, and their combination on the protein levels of HO-1 in LPS-stimulated macrophage cells, were also investigated. As shown in Figure 13, there was an highly increase of protein expression levels of HO-1, especially in full dose combination. In fact, the LUT and SFN combination increased HO-1 protein level to 6.20-fold that of the control.

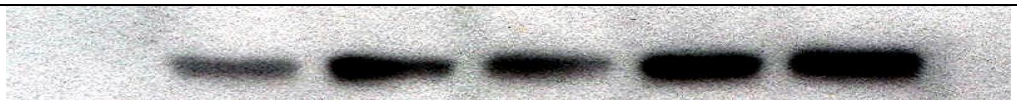
Anti-inflammatory effects of SFN have been studied in combination with other dietary components (curcumin and phenethyl isothiocyanate) in LPS-treated RAW 264.7 cells, and the results evinced synergistic interactions among these compounds in inhibiting inflammatory responses, such as elevated iNOS and COX-2 expression levels (Cheung et al., 2009).

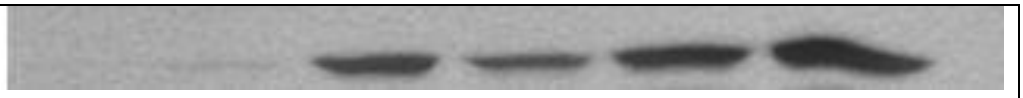
Moreover, Guo et al. (2012) has already verified the synergistic effects of nobiletin and SFN in LPS-stimulated RAW 264.7 cells. Similarly to the findings of the present study, the synergy between SFN and curcumin or phenethyl isothiocyanate or in combination with nobiletin displayed anti-inflammatory effects.

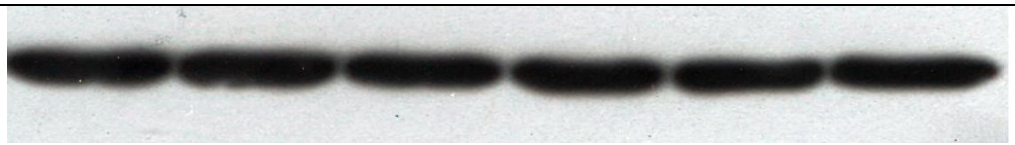
Figure 13: Inhibitory effects of LUT, SFN, and their combination on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 cells.. The standard deviations (all within ±15% of the means) were not shown. β-Actin served as an equal loading control. * indicates a statistical significance (*P* < 0.05, n = 3).

	CTL	LPS	LUT	SFN	HALF	FULL
LPS	-	+	+	+	+	+
LUT (μM)	-	-	15	-	7.5	15
SFN (μM)	-	-	-	1.2	0.6	1.2

iNOS						
		1.00	**0.33 ***	**0.18 ***	**0.24 ***	**0.12 ***

COX-2						
		1.00	**2.41***	**2.06 ***	**3.86 ***	**4.58 ***

HO-1						
	1.00	**0.88**	**3.42 ***	**2.47 ***	**4.31 ***	**6.20 ***

β-actin	

Conclusions

The main objective of this study was to determine the level to which LUT+TAN and LUT+SFN potentiate each other and produce enhanced inhibitory effects against inflammation. The results obtained demonstrated that combinations of LUT+TAN and LUT+SFN significantly inhibited NO production in LPS-stimulated macrophage cells, and these inhibitory effects were stronger than those produced by LUT, TAN or SFN alone at much higher doses. It is important to notice that all of the doses used for single and combination treatments were nontoxic to macrophage cells; this approach was follow to ensure that the inhibitory effects observed on NO production were due to inhibition on inflammation but not due to disruption of normal cellular function. The results showed that LUT+TAN combination inhibited LPS-induced protein upregulation of iNOS and COX-2, whereas the combination of LUT+SFN produced a stronger inhibition only on protein upregulation of iNOS and did not cause any decrease of COX-2 level, overall suggesting a synergistic effect. To determine if the enhanced inhibitory effects from both combinations (LUT+TAN and LUT+SFN) were synergistic or additive, the mode of interaction between LUT+TAN and LUT+SFN was analyzed by using an isobologram-based method. The results clearly demonstrated that both combinations produced synergistic inhibition on NO production in LPS-stimulated macrophage cells. In conclusion, the combination of LUT+TAN and LUT+SFN synergistically inhibited LPS-induced inflammation in RAW 264.7 macrophage cells, which was evidenced by lowered NO production and decreased iNOS, COX-2, and IL-1 levels. These effects were associated with synergistic induction of phase II enzyme HO-1 by both co-treatments. The results provide new knowledge and insights on the interactions between different dietary bioactive components, as well as on their biological effects, which is of outmost importance for the new trends on food product development.

6 CONCLUSION

6.1 Conclusion

Over the past few years, the concept of food has undergone a radical transformation, as it is not only characterized by sensory and nutritional properties, but it also plays an important role on human health status and on the reduction of the onset of many diseases.

Nowadays there is a high request of research on alternative rearing systems that are able to improve poultry welfare and to warrant high-quality and safe meat products. This thesis work was focused on the evaluation of the oxidative stability of poultry meats, obtained with different rearing systems, diets (supplemented with bioactive compounds), and packaging conditions.

In the first part of the thesis (chapter 2), it was evaluated the effects of rearing systems on the quality and oxidative stability of poultry meat. In general, a low level of both primary (PV=0.79-1.06 and 0.30-1.29 meq O_2/kg of lipid in breast and thigh, respectively) and secondary oxidation products (TBARs=0.06-0.19 and 0.10-0.13 mg MDA/kg of sample in breast and thigh, respectively) was found in chicken breast and thigh meat samples of two commercial categories (rotisserie and cut-up) obtained with different rearing systems (free-range (FR) and conventional (C)). Both oxidation parameters are far below the PV and TBARs levels associated with meat rancidity. However, the C system significantly increased peroxide value (PV) in Rotisserie thigh meat, whereas thiobarbituric acid reactive substances (TBARs) were significantly higher in Rotisserie breast meat obtained with the FR system. In the Cut-up category, no significant effects on the oxidation stability of thigh meat were detected, but the FR system led to a significantly higher TBARs content in breast meat. Despite these differences, it can be concluded that the two different rearing systems led to a low oxidation level in poultry meat, which ensures the oxidative quality of these food products

and their quality and safety from the consumer health standpoint. In the second part of the thesis (chapter 3), it was evaluated the effects of modified atmosphere packaging and rearing systems on poultry meat shelf-life. In general, a low level of both primary and secondary oxidation products was found in all chicken thigh meat samples obtained with two rearing systems (free-range (FR) vs. conventional (C)) and stored at 4°C for 14 days, using two packaging conditions (ordinary atmosphere (OA) vs. MAP (72% O_2 28% CO_2, modified atmosphere MA). PV ranged from 0.50 to 11.46 and from 0.47 to 8.28 meq O_2/kg of lipid in rotisserie and cut-up thigh meat, respectively, while TBARs varied from 0.11 to 1.24 and from 0.08 to 0.70 mg MDA/kg. Although the overall primary oxidation level of poultry meat was not significantly affected by rearing system, packaging conditions and storage time, secondary oxidation showed a significant increase in rotisserie category, obtained with FR breeding system and stored under ordinary packaging atmosphere. Moreover, long storage (10 and 14 days) would not be advisable under the tested conditions, as both commercial categories exhibited a significant TBARs increase. Therefore, it can be concluded that the two different rearing systems, in combination with ordinary or modified atmosphere packaging, led to a low oxidation level in poultry meat, but it is anyway advisable to avoid long-storage periods (over 7 days) under these conditions to ensure meat overall quality and consumer's acceptability.

In the third part of the thesis (chapter 4), it was evaluated the effects of feed supplementation with thymol, packaging and storage conditions on lipid oxidation of poultry meat. In general, a low level of both primary and secondary oxidation products was found in all chicken thigh meat samples obtained with different diets and packaging conditions during storage at 4°C for 14 days. PV and TBARs ranged from 0.47 to 12.9 meq O_2/kg of lipids and from 0.11 to 0.73 mg MDA/kg of sample, respectively. Packaging atmosphere (OA, and MA) and diet (A, E and F) did not significantly affect

the lipid content. Packaging with ordinary (OA) and modified atmosphere (MA, 72% O_2 28% CO_2) significantly ($p<0.05$) influenced the level of primary oxidation (O 0.47-12.99 meq O_2/kg lipid; M 1.12-12.45 meq O_2/kg lipid). However, no significant effects of the packaging atmosphere were observed on TBARs. Regarding secondary oxidation products, the statistical analysis shows some significant differences ($P<0.05$) on TBARs as related to storage time, in fact, all samples displayed a TBARs increase from 5 to 14 days. In conclusion, dietary supplementation with thymol extract at the tested levels did not provide further protection against lipid oxidation to poultry thigh meat stored under diverse packaging atmosphere (ordinary and modified atmosphere (MA, 72% O_2 28% CO_2). The direct addition of thyme to meat products may be another alternative that could lead to better oxidative stability than feeding supplementation of the thymol extract; however, its impact on the sensory profile of poultry and consumer's acceptance should be verified. These results demonstrated the importance of the purity and supplementation level of the bioactive compounds, as well as the great potentiality of their utilization in combination due to possible synergic effects. In the fourth part of the thesis (chapter 5), it was evaluated the health-promoting effects of bioactive food components: individual and combinatorial effects on the inhibition of inflammation. Several bioactive compounds showed individual anti-inflammatory effects, but only LUT+TAN and LUT+SFN had strong synergic effects. The main objective of this study was to determine the level to which LUT+TAN and LUT+SFN potentiate each other and produce enhanced inhibitory effects against inflammation. Noncytotoxic concentrations of LUT (3-15 μM), TAN (6-30 μM), SFN (0.24-1.2 μM) and their combinations were studied in LPS-stimulated RAW 264.7 macrophage cells. The results showed that both combination (LUT + TAN; LUT + SFN) treatments produced much stronger inhibitory effects on the production of nitric oxide (NO) than those compounds individually. The

results obtained demonstrated that combinations of LUT+TAN and LUT+SFN significantly inhibited NO production in LPS-stimulated macrophage cells, and these inhibitory effects were stronger than those produced by LUT, TAN or SFN alone at much higher doses. It is important to notice that all of the doses used for single and combination treatments were nontoxic to macrophage cells; this approach was follow to ensure that the inhibitory effects observed on NO production were due to inhibition on inflammation but not due to disruption of normal cellular function. The results showed that LUT+TAN combination inhibited LPS-induced protein upregulation of iNOS and COX-2, whereas the combination of LUT+SFN produced a stronger inhibition only on protein upregulation of iNOS and did not cause any decrease of COX-2 level, overall suggesting a synergistic effect. To determine if the enhanced inhibitory effects from both combinations (LUT+TAN and LUT+SFN) were synergistic or additive, the mode of interaction between LUT+TAN and LUT+SFN was analyzed by using an isobologram-based method. The results clearly demonstrated that both combinations produced synergistic inhibition on NO production in LPS-stimulated macrophage cells. In conclusion, the combination of LUT+TAN and LUT+SFN synergistically inhibited LPS-induced inflammation in RAW 264.7 macrophage cells, which was evidenced by lowered NO production and decreased iNOS, COX-2, and IL-1 levels. These effects were associated with synergistic induction of phase II enzyme HO-1 by both co-treatments. Overall, the results demonstrated for the first time that both combination (LUT + TAN; LUT + SFN) produced synergistic anti-inflammatory effects in LPS-stimulated macrophage cells. Furthermore, these results provide new knowledge and insights on the interactions between different dietary bioactive components, as well as on their biological effects, which is of outmost importance for the new trends on food product development.

7 REFERENCES

Anand P, Kunnumakara AB, Sundaram C, Harikumar BK, Tharakan ST, Lai OS, Sung B, and Aggarwal BB. 2008. Cancer is a preventable disease that requires major lifestyle changes. Pharmaceut Res. 25: 2097-2115.

Aggarwal BB, and Gehlot P. 2009. Inflammation and cancer: how friendly is the relationship for cancer patients? Curr Opin Pharmacol. 9: 351-369.

Arihara K. 2006. Strategies for designing novel functional meat products. Meat Sci. 74:219-229.

Aymerich T, Picouet PA, and Monfort JM. 2008. Decontamination technologies for meat products. Meat Sci 78:114-129.

Azain MJ. 2004. Role of fatty acids in adipocyte growth and development. J Anim Sci 82:916-924.

Aziza AE, Quezada N, and Cherian G. 2010. Feeding *Camelina sativa* meal to meat-type chickens: effect on production performance and tissue fatty acid composition. J Appl Poult Res 19:157-168.

Barbut S. 2001. Poultry products processing: an industry guide, edited by CRC Press LCC 2001, Chapter 1. ISBN: 978-1-58716-060-8.

Barroeta AC. 2007. Nutritive value of poultry meat: relationship between vitamin E and PUFA. World's Poult Sci J 63:277-284.

Bastide NM, Pierre FH, and Corpet DE. 2011. Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. Cancer Prev Res 4:177-184.

Betti M, Perez TI, Zuidhof MJ, and Renema RA. 2009a. Omega-3-enriched broiler meat: 3. Fatty acid distribution between triacylglycerol and phospholipid classes. Poult Sci 88:1740-1754.

Betti M, Schneider BL, Wismer WV, Carney VL, Zuidhof MJ, and Renema RA. 2009b. Omega-3-enriched broiler meat: 2. Functional properties, oxidative stability and consumer acceptance. Poult Sci 88:1085-1095.

Bingham S, and Riboli E. 2004. Diet and Cancer-the European prospective investigation into cancer and nutrition. Nat Rev 4:206-215.

Bogosavljevic-Boskovic S, Rakonjac S, Doskovic V, and Petrovic MD. 2012. Broiler rearing systems: a review of major fattening results and meat quality traits. World's Poultry Sci J. 68:217-228.

Bogosavljevic-Boskovic S, Mitrovic S, Djokovic R, Doskovic V, and Djermano V. 2010. Chemical composition of chicken meat produced in extensive indoor and free range rearing systems. Afr J Biotechnol 9:9069-9075.

Boros B, Jakabová S, Dörnyei A, Horváth G, Pluhár Z, Kilár F, and Felinger A. 2010. Determination of polyphenolic compounds by liquid chromatography-mass spectrometry in thymus species. J Chrom A 1217:7972-7980.

Boselli E, Cardenia V, and Rodriguez-Estrada MT. 2012. Cholesterol photosensitized oxidation in muscle foods. Eur J Lipid Sci Technol 114:644-655.

Boselli E, Caboni MF, Rodriguez-Estrada MT, Toschi-Gallina T, Daniel M, and Lercker G. 2005. Photoxidation of cholesterol and lipids of turkey meat during storage under commercial retail conditions. Food Chem 91:705-713.

Boselli E, Velazco V, Caboni MF, and Lercker G. 2001. Pressurized liquid extraction of lipids for the determination of oxysterols in egg-containing food. J Chromatogr A 917:239-244.

Botsoglou NA, Florou-Paneri P, Christaki E, Fletouris DJ, and Spais AB. 2002. Effect of dietary oregano essential oil on performance of chickens and on iron-induced lipid oxidation of breast, thigh and abdominal fat tissues. Brit Poult Sci 43:223-230.

Bou R, Codony R, Tres A, Decker EA, and Guardiola F. 2009. Dietary strategies to improve nutritional value, oxidative stability and sensory properties of poultry products. Crit Rev Food Sci 49:800-822.

Bou R, Guardiola F, Barroeta AC, and Codony R. 2005. Effect of dietary fat sources and zinc and selenium supplements on the composition and consumer acceptability of chicken meat. Poult Sci 84:1129–1140.

Brannan RG. 2009. Effect of grape seed extract on descriptive sensory analysis of ground chicken during refrigerated storage. Meat Sci 81:589-595.

Brenes A, Viveros A, Goni I, Centeno C, Sayago-Ayerdy SG, Arija I, and Saura-Calixto F. 2008. Effect of grape pomace concentrate and vitamin e on digestibility of polyphenols and antioxidant activity in chickens. Poult Sci 87:307-316.

Canivenc-Lavier MC, Brunold C, Siess MH, and Suschetet M. 1993. Evidence for tangeretin Odemethylation by rat and human liver microsomes. Xenobiotica 23:259–266.

Carlsen CU, Møller JKS, and Skibsted LH. 2005. Heme-iron in lipid oxidation. Coord Chem Rev 249:485-498.

Castellini C, Berri C, Le Bihan-Duval E, and Martino G. 2008. Qualitative attributes and consumer perception of organic and free-range poultry meat. World's Poult Sci J 64:500-513.

Castellini C, Dal Bosco A, Mugnai C, and Pedrazzoli M. 2006. Comparison of two chicken genotypes organically reared: oxidative stability and other qualitative traits of the meat. Ital J Anim Sci 5:355-368.

Castellini C, Mugnai C, and Dal Bosco A. 2002. Effect of organic production system on broiler carcass and meat quality. Meat Sci 60:219-225.

Cerella C, Sobolewski C, Dicato M, and Diederich M. 2010. Targeting COX-2 expression by natural compounds: a promising alternative strategy to synthetic COX-2 inhibitors for cancer chemoprevention and therapy. Biochem Pharmacol 80:1801-1815.

Chen X, Jiang W, Tan HZ, Xu GF, Zhang XB, Wei S, and Wang XQ. 2013. Effects of outdoor access on growth performance, carcass composition, and meat characteristics of broiler chickens. Poult Sci 92:435-443.

Chen D, Wan SB, Yang H, Yuan J, Chan TH, and Dou QP. 2011. EGCG, green tea polyphenols and their synthetic analogs and prodrugs for human cancer prevention and treatment. Adv Clin Chem. 53:155-177.

Chen CY, Peng WH, Tsai KD, and Hsu SL. 2007a. Luteolin suppresses inflammation-associated gene expression by blocking NF-Kb and AP-1

activation pathway in mouse alveolar macrophages. Life Sci 81:1602-1614.

Chen KH, Weng MS, and Lin JK. 2007b. Tangeretin suppresses IL-1β-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells. Biochem Pharmacol 73:215-227.

Cheung KL, Khor TO, and Kong AN. 2009. Synergistic effect of combination of phenethyl isothiocyanate and sulforaphane or curcumin and sulforaphane in the inhibition of inflammation. Pharm Res 26:224-231.

Chinnam N, Dadi PK, Sabri SA, Ahmad M, Kabir MA, and Ahmad Z. 2010. Dietary bioflavonoids inhibit *Escherichia coli* ATP synthase in a differential manner. Int J of Biol Macromol 46:478-486.

Colotta F, Allavena P, Sica A, Garlanda C, and Mantovani A. 2009. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30:1073-1081.

Coma V. 2008. Bioactive packaging technologies for extended shelf life of meat-based products. Meat Sci 78:90-103.

Cook NC, and Samman S. 1996. Review: flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. J Nutr Biochem 7:66-76.

Cooksey K. 2009. Active packaging and the shelf life of foods in food packaging and shelf life a practical guide, edited by Robertson GL. Chapter 20:367-381. 2010, Taylor and Francis. ISBN 978-1-4200-7844-2.

Corpet DE. 2011. Red meat and colon cancer: should we become vegetarians, or can we make meat safer? Meat Sci 89:310-316.

Cortinas L, Barroeta A, Villaverde C, Galobart J, Guardiola F, and Baucells MD. 2005. Influence of the dietary polyunsaturation level on chicken meat quality: lipid oxidation. Poult Sci 84:48-55.

Cortinas L, Villaverde C, Galobart J, Baucells M, Codony R, and Barroeta AC. 2004. Fatty acid content in chicken thigh and breast as affected by dietary polyunsaturation level. Poult Sci 83:1155-1164.

Coussens LM, and Werb Z. 2002. Inflammation and cancer. Nature 420:860-866.

Decker EA, and Xu Z. 1998. Minimizing rancidity in muscle foods. Food Technol 52:54-59.

De Kok TM, Van Breda SG, and Manson MM. 2008. Mechanisms of combined action of different chemopreventive dietary compounds. Eur J Nutr 47:51-59.

Demaria S, Pikarsky E, Karin M, Coussens LM, Chen YC, El-Omar EM, Trinchieri G, Dubinett SM, Mao JT, Szabo E, Krieg A, Weiner GJ, Fox BA, Coukos G, Wang E, Abraham RT, Carbone M, and Lotze MT. 2010. Cancer and inflammation: promise for biological therapy. J Immunother 33:335-351.

Demeyer D, Honikel K, and De Smet S. 2008. The World Cancer Research Fund report 2007. A challenge for the meat processing industry. Meat Sci 80:953-959.

Devlieghere F, Van Belle B, and Debevere J. 1999. Shelf life of modified atmosphere packed cooked meat products: a predictive model. Int J Food Microbiol 46:57-70.

Du M, and Ahn DU. 2002. Effect of dietary conjugated linoleic acid on the growth rate of live birds and on the abdominal fat content and quality of broiler meat. Poult Sci 81:428-433.

Eaton SB, Eaton SB, Konner MJ, and Shostak M. 1996. An evolutionary perspective enhances understanding of human nutritional requirements. J Nutr 126:1732-1740.

Erickson MC. 2002. Lipid oxidation of muscle foods, in food lipids chemistry nutrition and biotechnology, 2nd edition, edited by Akon CC and Min DB. Chapter 12. Dekker. ISBN:978-0-8247-0749-1.

Esterbauer H. 1993. Cytotoxicity and genotoxicity of lipid-oxidation products. Am J Clin Nutr 57:779S-786S.

EU-2004, Corrigendum of regulation EC/853/2004 on laying down specific hygienic rules for the food of animal origin, O J L 226, 26.06.2004, pp. 22-82.

European Commission. Allegate X.B. regulation L128/14. Off. J. Eur. Comm. 2002, May 15th 2002.

Fanatico AC, Pillai PB, Hester PY, Falcone C, Mench JA, Owens CM, and Emmert JL. 2008. Performance, livability, and carcass yield of slow- and fast-growing chicken genotypes fed low-nutrient or standard diets and raised indoors or with outdoor access. Poult Sci 87:1012-1021.

Fanatico A, Pillai P, Emmert J, and Owens C. 2007. Meat quality of slow-and fast-growing chicken genotypes fed low-nutrient or standard diets and raised indoors or with outdoor access. Poult Sci 86:2245-2255.

Fanatico AC, Cavitt LC, Pillai PB, Emmert JL, and Owens CM. 2005. Evaluation of slow-growing broiler genotypes grown with and without outdoor access: meat quality. Poult Sci 84:1785-1790.

Farmer LJ, Perry GC, Lewis PD, Nute GR, Piggott JR, and Patterson RLS. 1997. Responses of two genotypes of chicken to the diets and stocking densities of conventional UK and label rouge production systems. II. Sensory attributes. Meat Sci 47:77-93.

Faustman C, and Cassens RG. 1990. The biochemical basis for discoloration in fresh meat: a review. J Muscle Foods 1:217-243.

Fieser LF, Fieser M. Reagents for Organic Chemistry. John Wiley & Sons Inc.: New York, 1967; pp 191-192.

Fletcher DL. 2002. Poultry meat quality. World's Poult Sci J 58:131-135.

Floros JD, Newsome R, Fisher W, Barbosa-Cànovas GV, Chen H, Dunne CP, German JB, Hall RL, Heldman DR, Karwe MV, Knabel SJ, Labuza TP, Lund DB, Newell-McGloughlin M, Robinson JL, Sebranek JG, Shewfelt RL, Tracy WF, Weaver CM, and Ziegler GR. 2010. Feeding the world today and tomorrow: the importance of food science and technology. Compr Rev Food Sci Food Safety 9:572-599.

Folch J, Lees M, and Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509.

Formica JV, and Regelson W. 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33:1061-1080.

Friedman M, Henika PR, and Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and salmonella enterica. J Food Prot 10:1545-1560.

Gardner HW. 1989. Oxygen radical chemistry of polyunsaturated fatty acids. Free Rad Biol Med 7:65-86.

Garg ML, Sebokoba E, Wierzbicki AA, Thomson ABR, and Clandin MT. 1988. Differential effects of dietary linoleic acid and α-linolenic acid in rat tissue. Lipids 23:847-852.

German JB. 1990. Muscle lipids. J Muscle Foods 1:339-361.

Gill CO, and Gill AO. 2009. Packaging and the shelf life of fresh red and poultry meats in food packaging and shelf life a practical guide, edited by Robertson GL. Chapter 14:259-277. Taylor and Francis. ISBN 978-1-4200-7844-2.

Givens DJ, Kliem KE, and Gibbs RA. 2006. The role of meat as a source of n-3 polyunsaturated fatty acids in the human diet. Meat Sci 74:209-218.

Gonzalez CA, and Riboli E. 2010. Diet and cancer prevention: contributions from the European Prospective Investigation Into Cancer And Nutrition (EPIC) study. Eur J Cancer 46: 2555-2562.

Gonzalez-Esquerra R, and Leeson S. 2001. Alternatives for enrichment of eggs and chicken meat with n-3 fatty acids. Can J Anim Sci 81:295-305.

Grandison AS, and Jennings A. 1993. Extension of the shelf life of fresh minced chicken meat by electron beam irradiation combined with modified atmosphere packaging. Food Control 4:83-88.

Grashorn MA. 2007. Functionally of poultry meat. J Appl Poult Res 16:99-107.

Grivennikov S, Greten FR, and Karin M. 2010. Immunity, inflammation, and cancer. Cell 140:883-899.

Grunert KG, Bredahl L, and Brunsø K. 2004. Consumer perception of meat quality and implications for product development in the meat sector-a review. Meat Sci 66:259-272.

Gunstone FD, Harwood JL, and Padley FB. 1986. The Lipid Handbook. Chapman & Hall, London-New York. pp. 119-124, 453-457.

Guo S, Qiu P, Xu G, Wu X, Dong P, Yang G, Zheng J, McClements DJ, and Xiao H. 2012. Synergistic anti-inflammatory effects of nobiletin and sulforaphane in lipopolysaccharide-stimulated RAW 264.7 cells. J Agric Food Chem 60: 2157-2164.

Hämäläinen M, Nieminen R, Zaini Asmawi M, Vuorela P, Vapaatalo H, and Moilanen E. 2011. Effects of flavonoids on prostaglandin E2 production and on COX-2 and mPGES-1 expressions in activated macrophages. Planta Med 77:1504-1511.

Hanahan D, and Weinberg RA. 2000. The hallmarks of cancer. Cell 100:57-70.

Harris KG, Qian Y, Leonard SS, Sbarra DC, and Shi X. 2006. Luteolin and chrysin differentially inhibit cyclooxygenase-2 expression and scavenge reactive oxygen species but similarly inhibit prostaglandin-E2 formation in RAW 264.7 cells. J Nutr 136:1517-1521.

Hazell TJ. 1982. Iron and zinc compounds in the muscle meats of beef, lamb, pork and chicken. J Sci Food Agric 33:1049-1056.

Hermansen JE, Strudsholm K, and Horsted K. 2004. Integration of organic animal production into land use with special reference to swine and poultry. Livest Prod Sci 90:11-26.

Honikel KO. 2008. Composition and calories, in nutritional quality II, handbook of processed meats and poultry analysis, edited by Nollet LML, and Toldra F. Chapter 11:195-213. Taylor and Francis. ISBN: 978-1-4200-4531-4.

Hulan HW, Ackman RG, Ratnayake WMN, and Proudfoot FG. 1988. Omega-3 fatty acid levels and performance of broiler chickens fed redfish meal or redfish oil. Can J Anim Sci 68:533-547.

Jacxsens L, Devlieghere F, Van der Steen C, and Debevere J. 2001. Effect of high oxygen modified atmosphere packaging on microbial growth and sensorial qualities of fresh-cut produce. Int J Food Microbiol 71:197-210.

Jimènez-Colmenero F, Carballo J, and Cofrades S. 2001. Healthier meat and meat products: their role as functional foods. Meat Sci 59:5-13.

Kaefer CM, and Milner JA. 2008. The role of herbs and spices in cancer prevention. J Nutr Biochem 19:347-361.

Kamboh AA, and Zhu WY. 2013. Effect of increasing levels of bioflavonoids in broiler feed on plasma anti-oxidative potential, lipid metabolites, and fatty acid composition of meat. Poult Sci 92:454-461.

Kanner J. 2007. Dietary advanced lipid oxidation endproducts are risk factors to human health. Mol Nutr Food Res 51:1094-1101.

Kauffman RG. 2001. Meat composition, in meat science and applications, edited by Young OA, Rogers RW, Hui YH, and Nip WK. Chapter 1. Dekker. ISBN 978-0-8247-0548-0.

Kendrick J, and Watts BM. 1969. Acceleration and inhibition of lipid oxidation by heme compounds. Lipids 4:454-458.

Keokamnerd T, Acton JC, Han IY, and Dawson PL. 2008. Effect of commercial rosemary oleoresin preparations on ground chicken thigh meat quality packaged in a high-oxigen atmosphere. Poult Sci 87:170-179.

Kerry JP, O'Grady MN, and Hogan SA. 2006. Past, current and potential utilisation of active and intelligent packaging systems for meat and muscle-based products: a review. Meat Sci 74:113-130.

Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, Luo JL, and Karin M. 2009. Carcinoma produced factors activate myeloid cells via TLR2 to stimulate metastasis. Nature 457:102-106.

Kolakowska A. 2002. Lipid oxidation in food systems, in chemical and functional properties of food lipids, edited by Sikorski ZE, and Kolakowska A. Chapter 8. CRC Press. ISBN: 978-1-58716-105-6.

Kralik G, Ivanković S, Škrtić Z. 2005. Fatty acids composition of poultry meat produced in indoor and outdoor rearing systems. Agricult 11:38-42.

Labuza TP, Tsuyuki H, and Karel M. 1969. Kinetics of linoleate oxidation in model systems. J Am Oil Chem Soc 46:409-416.

Labuza TP, and Schmidl MK. 1988. Use of sensory data in the shelf life testing of foods: principles and graphical methods for evaluation. Cereal Food World 33:193-205.

Lai CS, Li S, Liu CB, Miyauchi Y, Suzawa M, Ho CT, and Pan MH. 2013. Effective suppression of azoxymethane-induced aberrant crypt foci formation in mice with citrus peel flavonoids. Mol Nutr Food Res *In press*.

Laury A, and Sebranek JG. 2007. Use of carbon monoxide combined with carbon dioxide for modifi ed atmosphere packaging of pre- and postrigor fresh pork sausage to improve shelf life. J Food Protect 70:937-942.

Lee DS. 2009. Packaging and the microbial shelf life of food in food packaging and shelf life a practical guide, edited by Robertson GL. Chapter 4:55-79. CRC Press. ISBN 978-1-4200-7844-2.

Lee KE, Kim HJ, An DS, Lyu ES, and Lee DS. 2008a. Effectiveness of modified atmosphere packaging in preserving a prepared ready-to-eat food. Packag Technol Sci 21:417-423.

Lee DS, Yam KL, and Piergiovanni L. 2008b. Food packaging science and technology. Boca Raton, Florida: CRC Press 397:424.

Lee DH, Anderson KE, Harnack LJ, Folsom AR, and Jacobs DR Jr. 2004. Heme iron, zinc, alcohol consumption, and colon cancer: Iowa women's health study. J Nat Cancer Inst 96:403-407.

Lee DS, Paik HD, Im GH, and Yeo IH. 2001. Shelf life extension of Korean fresh pasta by modified atmosphere packaging. J Food Sci Nutr 6:240-243.

Leusink G, Rempel H, Skura B, Berkyto M, White W, Yang Y, Rhee JY, Xuan SY, Chiu S, Silversides F, Fitzpatrick S, and Diarra MS. 2010. Growth performance, meat quality, and gut microflora of broiler chickens fed with cranberry extract. Poult Sci 89:1514-1523.

Li S, Pan MH, Lo CY, Tan D, Wang Y, Shahidi F, and Ho CT. 2009. Chemistry and health of polymethoxyflavones and hydroxylated polymethoxyflavones. J Function Foods I:2-12.

Lin WW, and Karin M. 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 117:1175-1183.

Liu RH. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr 78:517-520.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402-408.

Lopez-Bote CJ, Sanz Arias R, Rey AI, Castaño B, Isabel B, and Thos J. 1998. Effect of free-range feeding on n-3 fatty acid and α-tocopherol content and oxidative stability of eggs. Anim Feed Sci Tech 72:33-40.

Lu Y, Wu C. 2012. Reduction of *Salmonella enterica* on chicken breast by thymol, acetic acid, sodium dodecyl sulfate or hydrogen peroxide combinations as compared to chlorine wash. Int J Food Microbiol 152:31-34.

Lucera A, Mastromatteo M, Sinigaglia M, and Corbo MR. 2009. Combined effects of thymol, carvacrol and grapefruit seed extract on lipid oxidation and colour stability of poultry meat preparations. Int J Food Sci Technol 44:2256-2267.

Luna A, Labaque MC, Zygadlo JA, and Marin RH. 2010. Effects of thymol and carvacrol feed supplementation on lipid oxidation in broiler meat. Poult Sci 89:366-370.

Magdelaine P, Spiess MP, and Valceschini E. 2008. Poultry meat consumption trends in Europe. World's Poult Sci J 64:53-64.

Mantovani A. 2009a. Cancer inflaming metastasis. Nature 457:36-37.

Mantovani A. 2009b. Cancer-related inflammation: the seventh hallmark of cancer. American Society of Clinical Oncology (ASCO) Annual Meeting 723-726.

Mantovani A, Allavena P, Sica A, and Balkwill F. 2008. Cancer-related inflammation. Nature 454:436-444.

Mapiye C, Aldai N, Turner TD, Aalhus JL, Rolland DC, Kramer JKG, and Dugan MER. 2012. The labile lipid fraction of meat: from perceived disease and waste to health and opportunity. Meat Sci 92:210-220.

Meiyanto E, Hermawan A, and Anindyajati. 2012. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pac J Cancer Prev 13:427-436.

Micha R, Michas G, and Mozaffarian D. 2012. Unprocessed red and processed meats and risk of coronary artery disease and type 2 diabetes - an updated review of the evidence. Curr Atheroscler Rep 14:515-524.

Mosby TT, Cosgrove M, Sarkardei S, Platt KL, and Kaina B. 2012. Nutrition in adult and childhood cancer: role of carcinogens and anti-carcinogens. Anticancer Res 32:4171:4192.

Murakami A, and Ohnishi K. 2012. Target molecules of food phytochemicals: food science bound for the next dimension. Food Funct 3:462-476.

Narciso-Gaytán C, Shin D, Sams AR, Bailey CA, Miller RK, Smith SB, Leyva-Ovalle OR, and Sánchez-Plata MX. 2010a. Soybean, palm kernel, and animal-vegetable oils and vitamin E supplementation effect on lipid oxidation stability of sous vide chicken meat. Poult Sci 89:721-728.

Narciso-Gaytán C, Shin D, Sams AR, Bailey CA, Miller RK, Smith SB, Leyva-Ovalle OR, and Sánchez-Plata MX. 2010b. Dietary lipid source and vitamin E effect on lipid oxidation stability of refrigerated fresh and cooked chicken meat. Poult Sci 89:2726-2734.

Ozen T, Demirtas I, and Aksit H. 2011. Determination of antioxidant activities of various extracts and essential oil composition of *Thymus praecox* subsp. *skorpilii* var *skorpilii*. Food Chem 124:58-64.

Ozkececi RB, Karakaya M, Yilmaz MT, Saricoban C, and Ockerman HW. 2008. The effect of carcass part and packaging method on the storage stability of mechanically deboned chicken meat. J Muscle Foods 19:288-301.

Paine A, Eiz-Vesper B, Blasczyk R, and Immenschuh S. 2010. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 80:1895-1903.

Pan MH, Lai CS, Dushenkov S, and Ho CT. 2009. Modulation of inflammatory genes by natural dietary bioactive compounds. J Agric Food Chem 57:4467-4477.

Papazoglou S, Tsiraki M, and Savvaidis IN. 2012. Effect of thyme oil on the preservation of vacuum-packaged chicken liver. J Food Sci 77: M473:M480.

Park CM, Jin KS, Lee YW, and Song YS. 2011. Luteolin and chicoric acid synergistically inhibited inflammatory responses via inactivation of PI3K-Akt pathway and impairment of NF-κB translocation in LPS stimulated RAW 264.7 cells. Eur J Pharmacol 660:454-459.

Pearson AM, Love JD, and Shorland MA. 1977. Warmed-over flavor in meats, poultry and fish. Adv Food Res 23:1-74.

Petrou S, Tsiraki M, Giatrakou V, and Savvaidis IN. 2012. Chitosan dipping or oregano oil treatments, singly or combined on modified atmosphere packaged chicken breast meat. Int J Food Microbiol 156:264-271.

Pettersen MK, Nissen TE, and Nilsson A. 2004. Effect of packaging materials and storage conditions on bacterial growth, off-odour, pH and colour in chicken breast fillets. Packag Technol Sci 17:165-174.

Ponte PIP, Alves SP, Bessa RJB, Ferreira LMA, Gama LT, Bras JLA, Fontes CMGA, and Prates JAM. 2008. Influence of pasture intake on the fatty acid composition, and cholesterol, tocopherols, and tocotrienols content in meat from free-range broilers. Poult Sci 87:80-88.

Rababah TM, Hettiarachchy NS, and Horax R. 2004. Total phenolics and antioxidants activities of fenugreek, green tea, black tea and ginkgo extracts, vitamin E and tert-butylhydroquinone. J Agric Food Chem 52:5183-5186.

Rababah TM, Hettiarachchy NS, Horax R, Cho MJ, Davis B, and Dickson J. 2006. Thiobarbituric acid reactive substances and volatile compounds in chicken breast meat infused with plant extracts and subjected to electron beam irradiation. Poult Sci 85:1107-1113.

Ratnayake WMN, Ackman RG, and Hulan HW. 1989. Effect of redfish meal enriched diets on the taste and n-3 PUFA of 42-day-old broiler chickens. J Sci Food Agric 49:59-74.

Reiner GN, Labuckas DO, and García DA. 2009. Lipophilicity of some GABAergic phenols and related compounds determined by HPLC and partition coefficients in different systems. J Pharm Biomed Anal 49:686-691.

Rhee KSL, Anderson M, and Sams AR. 1996. Lipid oxidation potential of beef chicken, and pork. J Food Sci 61:8-12.

Ripoll G, Joy M, Muñoz F. 2011. Use of dietary vitamin E and selenium (Se) to increase the shelf life of modified atmosphere packaged light lamb meat. Meat Sci 87:88-93.

Robertson GL. 2009a. Food packaging and shelf life in food packaging and shelf life a practical guide, edited by Robertson GL. Chapter 1:1-16. Taylor and Francis. ISBN 978-1-4200-7844-2.

Robertson GL. 2009b. Food quality and indices of failure in food packaging and shelf life a practical guide, edited by Robertson GL. Chapter 2:17-30. Taylor and Francis. ISBN 978-1-4200-7844-2.

Robertson GL. 2006a. Food Packaging Principles and Practice, 2nd edition. Boca Raton, Florida: CRC Press, 291.

Sanosaka M, Minashima T, Suzuki K, Watanabe K, Ohwada S, Hagino A, Rose MT, Yamaguchi T, and Aso H. 2008. A combination of octanoate and oleate promotes in vitro differentiation of porcine intramuscular adipocytes. Comp Biochem Physiol 149:285-292.

Schönfeldt HC, and Gibson N. 2008. Changes in the nutrient quality of meat in an obesity context. Meat Sci 81:20-27.

Shahidi F. 2000. Antioxidants in food and food antioxidant. Nahrung 44:158-163.

Shantha NC, and Decker EA. 1994. Rapid, sensitive, iron-based spectrophotometric method for determination of peroxide values of food lipids. J AOAC Int 77:421-424.

Shay CM, Stamler J, Dyer AR, Brown IJ, Chan Q, Elliott P, Zhao L, Okuda N, Miura K, Daviglus ML, and Van Horn L. 2012. Nutrient and food intakes of middle-aged adults at low risk of cardiovascular disease: the international study of macro-/micronutrients and blood pressure (INTERMAP). Eur J Nutr 51:917-926.

Shirahigue LD, Plata-Oviedo M, De Alencar SM, Regitano D'Arce MAB, De Souza Vieira TMF, Cadorin Oldoni TL, and Contreras-Castillo CJ. 2010.

Wine industry residue as antioxidant in cooked chicken meat. Int J Food Sci Technol 45:863-870.

Siegford JM, Powers W, and Grimes-Casey HG. 2008. Environmental aspects of ethical animal production. Poult Sci 87:380-386.

Sikorski ZE, and Sinkiewicz I. 2010. The role of lipids in meat, in Chemical, biological, and functional aspects of foods lipids, 2nd edition, edited by Kolakowska A, Chapter 17:327-340. Taylor and Francis. ISBN: 978-1-4398-0237-3.

Simopoulos AP. 2008. The omega-6/omega-3 fatty acid ratio, genetic variation, and cardiovascular disease. Asia Pac J Clin Nutr 17:131-134.

Simopoulos AP. 2006. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: Nutritional implications for chronic diseases. Biomed Pharmacother 60:502-507.

Simopoulos AP. 2004. Omega-6/omega-3 essential fatty acid ratio and chronic disease. Food Rev Int 20:77-90.

Simopoulos AP. 2000. Symposium: role of poultry products in enriching the human diet with n-3 PUFA. Human requirement for n-3 polyunsaturated fatty acids. Poult Sci 79:961–970.

Simopoulos AP. 1999. Evolutionary aspects of omega-3 fatty acids in the food supply. Prostaglandins Leukot Essent Fatty Acids. 60:421-429.

Simopoulos AP. 1997. Omega-3 fatty acids in the prevention-mangement of cardiovascular disease. Can J Physiol Pharmacol 75:234-239.

Sivasothy Y, Sulaiman SF, Ooi KL, Ibrahim H, and Awang K. 2013. Antioxidant and antibacterial activities of flavonoids and curcuminoids from *Zingiber spectabile* Griff. Food Control 30:714-720.

Skandamis PN, and Nychas GJE. 2002. Preservation of fresh meat with active and modified packaging conditions. Int J Food Microbiol 79:35-45.

Slabyj BM, and Hultin HO. 1984. Oxidation of a lipid emulsion by a peroxidizing microsomal fraction from herring muscle. J Food Sci 49:1392-1393.

Smiddy M, Fitzgerald M, Kerry JP, Papkovsky DB, O'Sullivan CK, and Guilbeaut GG. 2002. Use of oxygen sensors for the non-destructive measurement of the oxygen content in modified atmosphere and vacuum packs of cooked chicken patties: impact of oxygen content on lipid oxidation. Meat Sci 61: 285-290.

Souci SW, Fachmann HJ, and Kraut A. 2008. Food composition and nutrition tables. 7th edition, Stuttgart: Medpharm Scientific Publ.

Spanou C, Veskoukis AS, Kerasioti T, Kontou M, Angelis A, Aligiannis N, Skaltsounis AL, and Kouretas D. 2012. Flavonoids glycosides isolated from unique legume plant extracts as novel inhibitors of xanthine oxidase. PloS One 7:1-7.

Tarladgis BG, Watts BM, Younathan MT, and Dugan LJr. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J Am Chem Soc 37:44-48.

Teixeira B, Marques A, Ramos C, Neng NR, Nogueira JMF, Saraiva JA, and Nunes ML. 2013. Chemical composition and antibacterial and antioxidant properties of commercial essential oils. Ind Crop Prod 43:587-595.

Trichopoulou A, Soukara S, and Vasilopoulou E. 2007. Traditional foods: a science and society perspective. Trends Food Sci Tech 18:420-427.

USDA. 2007. USDA's National Organic Program. United States Department of Agriculture. http://www.ams.usda.gov/ NOP/FactSheets/Backgrounder.html Accessed Aug. 2007.

Vainio H, and Weiderpass E. 2006. Fruit and Vegetables in Cancer Prevention Nutr. Cancer 54:111-142.

Van Boekel MAJS. 2008. Kinetic modeling of food quality: a critical review. Compr Rev Food Sci Food Safety 7:144-158.

Van Horne PLM, and Achterbosch TJ. 2008. Animal welfare in poultry production systems: impact of EU standards on world trade. World's Poultry Sci J 64:40-52.

Velasco S, Ortiz LT, Alzueta C, Rebolé A, Treviño J, and Rodríguez ML. 2010. Effect of inulin supplementation and dietary fat source on performance, blood serum metabolites, liver lipids, abdominal fat

deposition, and tissue fatty acid composition in broiler chickens. Poult Sci 89:1651-1662.

Vihavainen EJ, and Bjorkroth KJ. 2007. Spoilage of value-added, high-oxygen modified-atmosphere packaged raw beef steaks by *Leuconostoc gasicomitatum* and *Leuconostoc gelidum*. Int J Food Microbiol 119:340-345.

Vila R. 2002. Flavonoids and further polyphenols in the genus *Thymus* in Thyme: the Genus Thymus, edited by Stahl-Biskup E and Saez F. Chapter 5. 2002, Taylor and Francis. ISBN 978-0-415-28488-2.

Wallace RJ, Oleszek W, Franz C, Hahn I, Baser KHC, Mathe A, and Teichmann K. 2010. Dietary plant bioactives for poultry health and productivity. Brit Poult Sci 51:461-487.

Wang KH, Shi SR, Dou TC, and Sun HJ. 2009. Effect of a free-range raising system on growth performance, carcass yield, and meat quality of slow-growing chicken. Poult Sci 88:2219-2223.

Wang ML, Suo X, Gu JH, Zhang WW, Fang Q, and Wang X. 2008. Influence of grape seed proanthocyanidin extract in broiler chickens: Effect on chicken coccidiosis and antioxidant status. Poult Sci 87:2273-2280.

Wang MY, and Brown WD. 1983. Effects of elevated CO_2 atmosphere on storage of freshwater cray fish (Pacifastacus leniusculus). J Food Sci 48:158-162.

Wang SQ, Zhu XF, Wang XN, Shen T, Xiang F, and Lou HX. 2013. Flavonoids from *Malus hupehensis* and their cardioprotective effects against doxorubicin-induced toxicity in H9c2 cells. Phytochem 87:119-125.

Whitmore BB, and Naidu AS. 2000. Glucosinolates in natural food antimicrobial systems, edited by Naidu AS. Chapter 15. 2000, CRC Press. ISBN: 978-0-8493-2047-7.

Williamson CS, Foster RK, Stanner SA, and Buttriss JL. 2005. Red meat in the diet. Nutr Bull 30:323-355.

Wong MCY, Emery PW, Preedy VR, and Wiseman H. 2008. Health benefits of isoflavones in functional foods? Proteomic and metabonomic advances. Inflammopharmacol 16:235-239.

Wood JD, Enser M, Fisher AV, Nute GR, Sheard PR, Richardson RI, Hughes SI, and Whittington FM. 2008. Fat deposition, fatty acids composition and meat quality: a review. Meat Sci 78:343-358.

Wood JD, Enser M, Richardson RI, and Whittington FM. 2007. Fatty acids in meat and meat products, in fatty acids in foods and their implications, third edition, edited by Chow CK. Chapter 5:87-107. Taylor and Francis. ISBN: 978-0-8493-7261-2.

Wood JD, Richardson RI, Nute GR, Fisher AV, Campo MM, Kasapidou E, Sheard PR, and Enser M. 2003. Effects of fatty acids on meat quality: a review. Meat Sci 66:21-32.

Xagorari A, Papapetropoulos A, Mauromatis A, Economou M, Fotsis T, and Roussos C. 2001. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. J Pharmacol Exp Ther 296:181-187.

Xiao H. 2009. Potential enhancement of cancer inhibition by using statins in combination with pharmacological and dietary agents. Cancer Chemoth Rev 4:183-192.

Xiao H, Yang CS, Li S, Jin H, Ho CT, and Patel T. 2009. Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. Mol Nutr Food Res 53:398-406.

Xiao H, and Yang CS. 2008a. Combination regimen with statins and NSAIDs: a promising strategy for cancer chemoprevention. Int J Cancer 123:983-990.

Xiao H, Zhang Q, Lin Y, Reddy BS, and Yang CS. 2008b. Combination of atorvastatin and celecoxib synergistically induces cell cycle arrest and apoptosis in colon cancer cells. Int J Cancer 122:2115-2124.

Yam KL, Takhistov PT, and Miltz J. 2005. Intelligent packaging: concepts and applications. J Food Sci 70: R1-10.

Yang CS, Landau JM, Huang MT, and Newmark HL. 2001. Inhibition of cancerogenesis by dietary polyphenolic compounds. Annu Rev Nutr 21:381-406.

Yanishlieva NV, Marinova EM, Gordon MH, and Raneva VG. 1999. Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. Food Chem 64:59-66.

Yao LH, Jiang YM, Shi J, Tomàs-Barberàn FA, Datta N, Singanusong R, and Chen SS. 2004. Flavonoids in food and their health benefits. Plant Food Hum Nutr 59:113-122.

Ye CL, Dai DH, and Hu WL. 2013. Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa* L.). Food Control 30:48-53.

Yeung RMW, and Morris J. 2001. Consumer perception of food risk in chicken meat. Nutr Food Sci 6:270-278.

Zhang G, He L, and Hu M. 2011. Optimized ultrasonic-assisted extraction of flavonoids from *Prunella vulgaris* L. and evaluation of antioxidant activities in vitro. Inn Food Sci Emerg Technol 12:18-25.

Zhang Y. 2004. Cancer Chemoprevention with sulforaphane, a dietary isothiocyanate in phytochemicals in health and disease, edited by Bao Y and Fenwick R. Chapter 6:121-141. 2004, Dekker Marcel. ISBN 978-0-8247-4023-8.

Zhang Y, Talalay P, Cho CG, and Posner GH. 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 89:2399-2403.

Zhang W, Xiao S, Samaraweera H, Lee EJ, and Ahn DU. 2010. Improving functional value of meat products. Meat Sci 86:15-31.

http://www.fao.org/docrep/013/al714e/al714e00.pdf

http://www.nal.usda.gov/fnic/foodcomp/Data/Other/EB03_VegFlav.pdf

www.thepoultrysite.com

www.unionenazionaleavicoltura.it