

In Vitro Effects of Cooking Methods on Digestibility of Lipids and Formation of Cholesterol Oxidation Products in Pork

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Abstract

This study investigated the effects of cooking methods on the digestibility of lipids and formation of cholesterol oxidation products (COPs) in pork, during *in vitro* human digestion. Pork patties were cooked using four different methods (oven cooking, pan frying, boiling, and microwaving), to an internal temperature of approximately 85°C. The digestibility of pork patties were then evaluated, using the *in vitro* human digestion model that simulated the composition (pH, minerals, surface-active components, and enzymes) of digestive juices in the human mouth, stomach, and small intestine. The total lipid digestibility was higher after microwave cooking, whereas pan-frying resulted in lower *in vitro* digestibility, compared to the other cooking methods. The microwaving method followed by *in vitro* digestion also showed significantly higher content of free fatty acids and thiobarbituric acid reactive substances (TBARS), compared to the other cooking methods; whereas, the pan frying and boiling methods showed the lowest. Cholesterol content was not significantly different among the cooked samples before, and after *in vitro* human digestion. The formation of COPs was significantly higher in the microwave-treated pork samples, compared to those cooked by the other methods, which was consistent with the trend for lipid peroxidation (TBARS). We propose that from the point of view of COPs formation and lipid oxidation, the pan-frying or boiling methods would be useful.

Keywords: cooking methods, *in vitro* human digestion, lipid digestibility, lipid oxidation, cholesterol oxidation products

Introduction

Lipid oxidation occurring in meat during cooking is the process behind the production of volatile substances that account for taste and odor of the cooked food (Broncano *et al.*, 2009). However, excessive lipid peroxidation during cooking process can also lead to a loss of meat nutritional value mainly due to formation of cholesterol oxidation products (COPs) (Hur *et al.*, 2007). It is known that changes in food quality during cooking correlate with temperature and cooking times (Broncano *et al.*, 2009) and that extended exposure to high temperatures can generate highly oxidized products, which can negatively affect food quality (Kesava Rao *et al.*, 1996). Our previous study showed that microwave cooking promoted lipid oxidation and formation of COPs (Hur *et al.*, 2004). Oxi-

dized lipids including cholesterol not only affect food nutritional value, but have also been related to different adverse effects such as cytotoxicity, atherogenicity, mutagenicity, and carcinogenicity (Hur *et al.*, 2013). Several studies have demonstrated the involvement of COPs in the rapid progression of coronary heart disease and atherosclerosis: COPs were shown to cause endothelial cell damage, which initiated complex series of pathological events that ultimately led to plaque formation (Li and Mehta, 2005; Poli *et al.*, 2009; Sevanian *et al.*, 1995). In most animals COPs are used as intermediate substances in the endogenous synthesis of several molecules such as bile acids and steroid hormones, and ingestion of the COPs-containing foods can increase their concentration in plasma (Vicente and Torres, 2007). Although many studies reported production of oxidized lipids including cholesterol in cooked meats, the influence of cooking methods on lipid digestibility and COPs formation during digestion remains to be elucidated. The purpose of this study was to investigate the effects of cooking methods on the digestibility of lipids and formation of COPs in

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pork patties during *in vitro* human digestion.

Materials and Methods

Sample preparation

Pork loins were ground twice through a 3 mm-mincer plate, mixed with back fat (up to 20%) for 5 min and used to make 100 g patties (approximately 9 cm in diameter, 1.5 cm thick). Patties were cooked by four different methods (oven cooking, pan frying, boiling, and microwaving) to an internal temperature of 85-90°C.

1. *Oven cooking*: Patties were placed in a 2 cm deep pan and heated in convection oven set to 180°C. Heat was generated by a heating bar and each patty was turned over twice in order to cook top and bottom surfaces. During cooking, samples were heated initially for 5 min on each side followed by a further 10 min per side. After heat treatment, patties were cooled to and held at 5°C or lower for 5 min.

2. *Pan frying*: Patties were placed on an electric pan (30 cm diameter, 5 cm depth) and cooked at 180°C for a total of 5 min (2 min 30 s each side). To stabilize the temperature of the iron pan, it was preheated for 5 min before adding of the sample. After heat treatment, patties were cooled to and held at 5°C or lower for 5 min.

3. *Boiling*: Patties were put into a zipper bag, then gently squeeze out the air and dipped into boiling water (water bath) for 5 min. After heat treatment, patties were cooled to and held at 5°C or lower for 5 min.

4. *Microwaving*: Patties were placed on a glass dish and

cooked in a microwave oven (3,000 MHz) with turning over twice between cycles. Core temperature was measured with a flexible internal thermometer (Temp 300, Thermo Scientific, USA). The cooked samples were vacuum packaged in an oxygen-proof PVDC bag and stored at 0-4°C until use.

In vitro human digestion model

We used a modified *in vitro* human digestion model described in previous studies (Hur *et al.*, 2009; Versantvoort *et al.*, 2005). The model imitated the following digestive stages:

- I. *Pre-Ingestion*: pork patties.
- II. *Mouth*: 5 g of pork patties were mixed with 6 mL of simulated saliva fluid (pH 6.8) and stirred for 5 min at 37°C.
- III. *Stomach*: 12 mL of simulated gastric fluid (pH 2) was added and the mixture was stirred for 2 h at 37°C.
- IV. *Small Intestine*: 12 mL of duodenal juice, 6 mL of bile juice, and 2 mL of sodium bicarbonate solution (pH 6.5-7) were added, and the mixture was stirred for 2 h at 37°C.

Composition of the simulated saliva, gastric, duodenal, and bile fluids are listed in Table 1. During the *in vitro* digestion, process samples were swirled at 60 rpm in a shaking water bath (Model HB-205SW, Hanbaek, Co., Korea) to simulate gastrointestinal tract motility.

Total lipid digestibility

The digestibility of total lipids in the pork patties dur-

Table 1. Composition of various synthetic juices used in *in vitro* human digestion model

	Saliva	Gastric juice	Duodenal juice	Bile juice
Inorganic components	10 ml KCl 89.6 g/l	15.7 ml NaCl 175.3 g/l	40 ml NaCl 175.3 g/l	30 ml NaCl 175.3 g/l
	10 ml KSCN 20 g/l	3.0 ml NaH ₂ PO ₄ 88.8 g/l	40 ml NaHCO ₃ 84.7 g/l	68.3 ml NaHCO ₃ 84.7 g/l
	10 ml NaH ₂ PO ₄ 88.8 g/l	9.2 ml KCl 89.6 g/l	10 ml KH ₂ PO ₄ 8 g/l	4.2 ml KCl 89.6 g/l
	10 ml NaSO ₄ 57 g/l	18 ml CaCl ₂ ·2H ₂ O 22.2 g/l	6.3 ml KCl 89.6 g/l	150 µl HCl 37%/g
	1.7 ml NaCl 175.3 g/l	10 ml NH ₄ Cl 30.6 g/l	10 ml MgCl ₂ 5 g/l	
	20 ml NaHCO ₃ 84.7 g/l	6.5 ml HCl 37%/g	180 µl HCl 37%/g	
Organic components	8 ml urea 25 g/l	10 ml glucose 65 g/l	4 ml urea 25 g/l	10 ml urea 25 g/l
		10 ml glucuronic acid 2 g/l		
		3.4 ml urea 25 g/l		
		10 ml glucosamine hydrochloride 33 g/l		
Add to mixture of organic+ inorganic components	290 mg α-amylase	1 g BSA	9 ml CaCl ₂ ·2H ₂ O 22.2 g/l	10 ml CaCl ₂ ·2H ₂ O 22.2 g/l
	15 mg uric acid	2.5 g pepsin	1 g BSA	1.8 g BSA
	25 mg mucin	3 g mucin	9 g pancreatin	30 g bile
		1.5 g lipase		
pH	6.8±0.2	1.30±0.02	8.1±0.2	8.2±0.2

For all inorganic and organic solutions, distilled water was added up to 500 mL. If necessary, pH of the juices was adjusted to the appropriate level.

ing simulated human gastrointestinal digestion was determined by the rates of infiltration through dialysis tubing and expressed as percentage of total lipids inside and outside dialysis bags. Briefly, the dialysis tubing was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, USA) with a molecular weight cut-off of 12-14 kDa. The dialysis tubing was cut into pieces of 10 cm and soaked in distilled water at 4°C before usage. After stomach digestion, the sample was then transferred to dialysis bags with duodenal juice, bile juice and sodium bicarbonate solution. The dialysis tubes with digestive samples were placed into a sodium phosphate buffer (pH 6.9) and then incubated at 37°C for 2 h in a stirred water bath.

Digestibility (%) = (lipid concentration inside the dialysis tube – lipid concentration outside the dialysis tube) / (lipid concentration inside the dialysis tube) × 100

Total lipid content

Total lipids were extracted with chloroform and methanol as previously described (Folch *et al.*, 1957). Each meat sample (5 g) was combined with 50 mL of Folch reagent (chloroform/methanol, 2:1 v/v) and 50 mL of 10% butylhydroxyanisole and homogenized using a Polytron homogenizer (IKA, Model T25, Germany) for 10 s. The homogenate was filtered through Whatman No. 1 filter paper. The remaining sediment and filter paper were blended with 50 mL of Folch reagent and refiltered. Distilled water (25 mL) was added to the filtered extract and the mixture was centrifuged at 500 g for 10 min. The upper layer of methanol and water was removed with an aspirator, and the bottom chloroform layer with extracted lipids was passed through anhydrous sodium sulfate (Na₂SO₄) which then was rinsed with 30 mL of chloroform. The extracts were concentrated at 40°C using an evaporator (Zymarkturbovap 500, USA).

Free fatty acid content

Free fatty acid content was determined by the modified AOAC method (AOAC, 1995) and quantified by titrimetry as the amount of KOH required to neutralize carboxylic acid in 1 g of sample. Pork patty samples (5 g) were homogenized in 50 mL test tubes with 15 mL of deionized distilled water at 1000 g for 10 s using a Polytron homogenizer. The homogenate (2 mL) was transferred to a 300 mL flask, and 100 mL of ether/ethanol mixture (1:1, v/v) was added. Several drops of phenolphthalein were added and free fatty acid content was determined by titration with 0.1 M KOH according to the equation:

$$\text{free fatty acids (KOH/g)} = 5.611 \times A \times F / \text{sample weight (g)}$$

where A is the volume of 0.1 M KOH (mL) and F is the titer of KOH.

Thiobarbituric acid-reactive substances (TBARS)

TBARS content was determined by the modified method described previously (Buege and Aust, 1978). Samples of pork patties (5 g) pre- and post-digestion were placed in 50 mL test tubes and homogenized with 15 mL of deionized distilled water using a Polytron homogenizer at 1000 g for 10 s. One mL of pork patty homogenate was transferred to a disposable test tube (3×100 mm), and 50 µL of 10% butylated hydroxyanisole and 2 mL thiobarbituric acid/trichloroacetic acid (TBA/TCA) were added. The mixture was vortexed and incubated in boiling water for 15 min to develop color. Samples were cooled in cold water for 5 min, vortexed, and centrifuged for 15 min at 2000×g. The absorbance of the resulting supernatant was determined at 531 nm against a blank containing 1 mL of deionized distilled water and 2 mL of TBA/TCA solution. TBARS were calculated based on a standard curve of malondialdehyde (MA), freshly prepared by acidification of 1,1,3,3-tetraethoxypropane in the range between 0.02 and 0.3 µg/mL ($y = 0.8729x + 0.0382$, $r = 0.9961$) and expressed as mg of MA per kg of sample.

Cholesterol oxidation products

COPs formation in samples was determined as previously described (Ahn *et al.*, 1999). The dried lipid samples (Folch *et al.*, 1957) were dissolved in hexane to concentration of 0.1g fat/mL hexane. Silicic acid (100 mesh), celite-545, and CaHPO₄ were mixed at 10:9:1 (w/w/w) in chloroform and packaged into a glass column. The columns were washed with 10 mL of hexane/ethyl acetate (9:1, v/v) and 0.2 g of lipid samples in hexane was added with an internal standard (5α-cholestane) and loaded onto the silicic acid column. Neutral lipids, phospholipids, and cholesterol were eluted by hexane/ethyl acetate (9:1, v/v) and hexane/ethyl acetate/diethyl ether (4:1:2, v/v/v). COPs were eluted with acetone/ethyl acetate/methanol (10:10:1) and dried under nitrogen. The dried COPs were mixed with 200 µL pyridine and 100 µL bis-[trimethylsilyl]trifluoroacetamide + 1% trimethylchlorosilane, derivatized by heating at 80°C for 1h in a dry bath, and analyzed using a gas chromatograph (Agilent 6890, USA) equipped with an on-column capillary injector and a flame ionization detector. A ramped oven temperature conditions

(180°C for 2.5 min, increased to 230°C at 2.5°C/min, then held at 230°C for 7.5 min) were used. Temperature of both the inlet and detector was 280°C. Helium was the carrier gas with linear flow at 1.1 mL/min. Detector air, H₂, and make-up gas (He) flows were 350, 35, and 43 mL/min, respectively. Six COPs were analyzed (7 α -hydroxycholesterol, 7 β -hydroxycholesterol, 7-ketocholesterol, 20 α -hydroxycholesterol, cholestanetriol, and 25-hydroxycholesterol) to assess total COPs amount. COPs were identified by comparison of retention times with those of known standards. Relative quantities were expressed as percent (w/w) of total COPs.

Statistical analysis

Statistical analyses were conducted for three batches of pork patties. Analyses of variance (ANOVA) were performed to test the effects of cooking methods on pork digestibility, free fatty acids content, lipid oxidation, and COPs formation using SAS software (SAS Inst. Inc., USA). Data were expressed as the mean (n=15) with standard deviation (SD) and differences between samples were considered significant at $p < 0.05$.

Results

Effects of cooking methods on the digestibility of total lipids are shown in Fig. 1. In *in vitro* human digestion model, total lipid digestibility was significantly higher in the microwave-cooked samples, whereas the pan-fried patties showed significantly lower total lipid digestibility compared with those cooked by the other methods.

Effects of cooking methods on free fatty acid content are shown in Fig. 2. All cooking methods resulted in increased levels of free fatty acids in the digested sam-

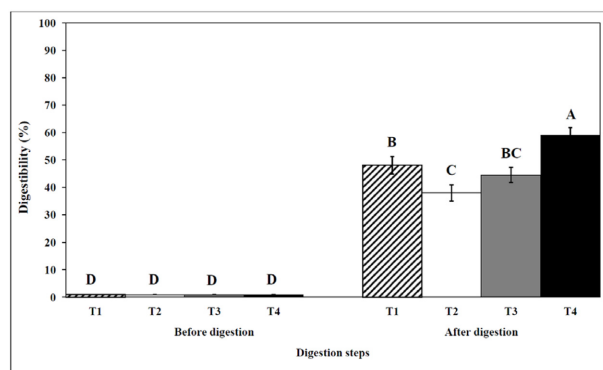


Fig. 1. Effect of cooking methods on total lipid digestibility in pork patties subjected to *in vitro* human digestion. Cooking methods: oven (T1), pan-frying (T2), boiling (T3), microwave heating (T4).

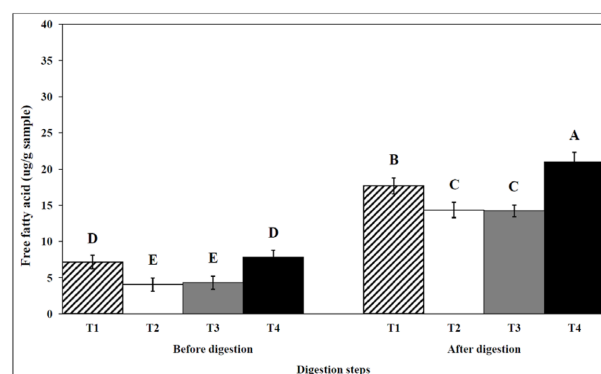


Fig. 2. Effect of cooking methods on the free fatty acid contents in pork patties subjected to *in vitro* human digestion. Cooking methods: oven (T1), pan-frying (T2), boiling (T3), microwave heating (T4).

ples. However, patties cooked in the microwave showed significantly higher free fatty acid content after *in vitro* digestion than those cooked by frying or boiling. The release of free fatty acids indicates that lipids including cholesterol were processed by digestive enzymes and bile salts during *in vitro* digestion. In this regard, higher free fatty acid content in the *in vitro* digested patties reflected increases in total lipid digestibility.

Effects of cooking methods on TBARS levels are shown in Fig. 3. TBARS formation is an indicator of lipid peroxidation and it was increased after *in vitro* human digestion in all cooked samples. Similar to free fatty acid levels, TBARS values were significantly higher in the patties subjected to *in vitro* digestion after microwaving and oven cooking, whereas pan-fried and boiled samples had lower TBARS levels.

Effects of cooking methods on cholesterol content are shown in Fig. 4. In all the samples cholesterol levels decreased significantly after *in vitro* digestion, possibly

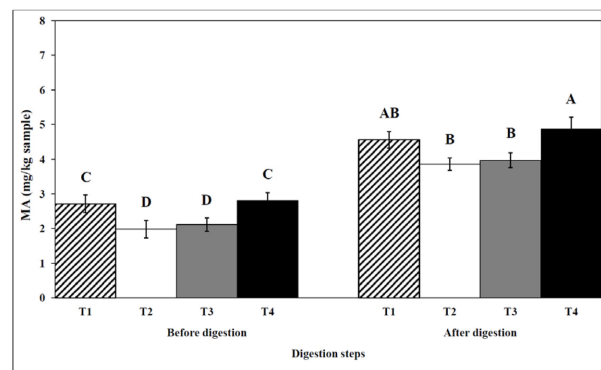


Fig. 3. Effect of cooking methods on TBARS concentration in pork patties subjected to *in vitro* human digestion. Cooking methods: oven (T1), pan-frying (T2), boiling (T3), microwave heating (T4).

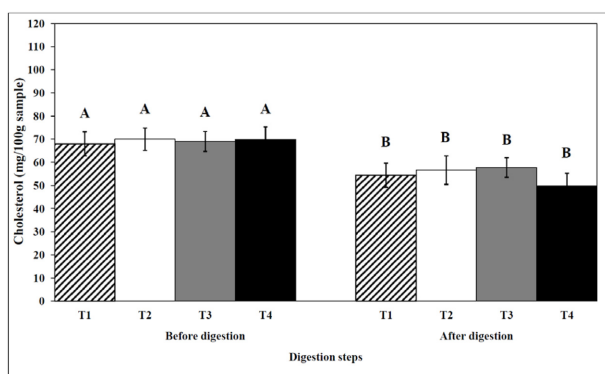


Fig. 4. Effect of cooking methods on the cholesterol contents in pork patties subjected to *in vitro* human digestion. Cooking methods: oven (T1), pan-frying (T2), boiling (T3), microwave heating (T4).

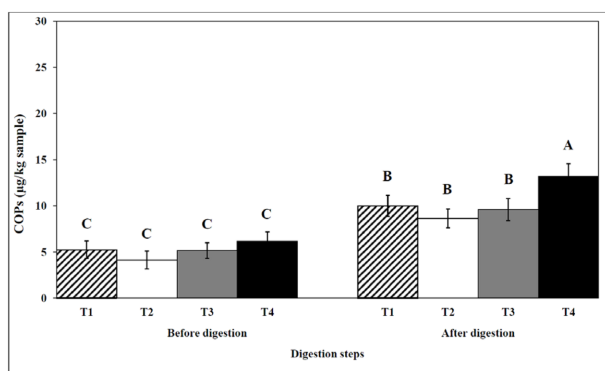


Fig. 5. Effect of cooking methods on COP concentration in pork patties subjected to *in vitro* human digestion. Cooking methods: oven (T1), pan-frying (T2), boiling (T3), microwave heating (T4).

owing to cholesterol's oxidization to COPs. However, cholesterol content in both undigested and digested patties was not significantly affected by cooking methods.

Effects of cooking methods on the total production of COPs are shown in Fig. 5. Before human digestion, the amount of total COPs did not vary significantly among the differently cooked samples although the levels of individual cholesterol oxides showed some changes depending on the cooking method. However, after *in vitro* digestion, the amount of total COPs was significantly higher in the microwave-cooked patties than in the other samples.

Discussion

Our results show that total lipid digestibility, lipid oxidation, cholesterol levels, and COPs production in samples subjected to human digestion *in vitro* were influ-

enced by cooking methods. Total lipid digestibility, free fatty acid levels, lipid oxidation, and COPs formation were the highest in the samples cooked in microwave, followed by oven cooking, boiling, and pan-frying. It is well known that lipid oxidation occurs because of high temperature treatment during cooking and the degree of oxidation depends on cooking methods, processing temperature and time. We hypothesized that cooking methods influence total lipid digestibility and that the increased lipid oxidation is probably related to the damage of the cell membrane caused by heating. Several studies (Hernández *et al.*, 1999; Rodriguez-Estrada *et al.*, 1997) have shown that roasting, which is based on high temperature treatment during a long time, is characterized by increased lipid oxidation compared to other methods. It has been reported that cooking in microwave caused high lipid oxidation because the process promoted oxidative reactions (Rodríguez-Estrada *et al.*, 1997). In this study, the microwave heating also resulted in higher levels of lipid oxidation (measured by TBARS production) and COPs generation and increased free fatty acid release compared to the other cooking methods, probably because the meat is more evenly heated than generally occurs in other cooking techniques. In contrast, during pan frying the heat is gradually conducted from the meat surface to the core, which is less efficient than heating in the microwave. This difference in thermal conduction may be the main reason why *in vitro* digestion resulted in higher total lipid digestibility and COPs contents for the microwave-treated patties than for the pan-fried samples. COPs formation in the cooked pork patties is promoted by high temperature treatments (Broncano *et al.*, 2009; Conchillo *et al.*, 2003; Grau *et al.*, 2001). Several studies (Broncano *et al.*, 2009; Chan *et al.*, 1993) reported that heating of cholesterol-containing food increases COPs production. In this study, microwave treatment increased temperature in the pork patties more rapidly compared to the other cooking methods (data are not shown). The microwaving resulted in the highest levels of lipid digestibility and total COPs in the pork patties after *in vitro* digestion compared to the other cooking methods. In general, microwave-based heating is an extremely rapid method to cook meats. Heating is generated by electromagnetic radiation causing polarized molecules in the food to rotate and build up thermal energy by intermolecular interactions, therefore, the microwave method should more easily damage the cholesterol structure than other cooking methods.

Our results demonstrate that the rate of cholesterol oxidation in pork patties is considerably accelerated after

human digestion *in vitro*, and appears to follow the same trend as lipid oxidation. These results are consistent with previous findings showing positive correlation between COPs and TBARS production (Broncano *et al.*, 2009). In an early work of Smith *et al.* (1987), it was postulated that the hydroperoxides of polyunsaturated fatty acid formed during lipid oxidation may promote cholesterol oxidation. Unsaturated fatty acids with one or more double bonds between carbon atoms are chemically unstable and prone to oxidation (Hur *et al.*, 2007), including that by diatomic molecular oxygen. The hydroperoxides of unsaturated fatty acid initiate oxidation of cholesterol. It has been reported (Chan *et al.*, 1993) that lipid radicals formed during food processing and storage can accelerate the formation of COPs. Therefore, COPs production should correlate with lipid peroxidation and generation of free radicals from unsaturated fatty acids. Prevention of lipid oxidation during cooking may therefore result in decrease of cholesterol oxidation and COPs formation.

In the stomach, lipids and cholesterol are exposed to highly acidic environment (pH 1-3) and subjected to enzymatic hydrolysis by gastric lipases. In the small intestine, partially hydrolyzed cholesterol is exposed to digestive juices containing cholesterol esterase; mixing of the chyme with alkali digestive juices containing bile salts and sodium bicarbonate causes elevation of pH levels to neutral which contributes to cholesterol oxidation (Hur *et al.*, 2013). In our study, lipid oxidation and the release of free fatty acids indicate that lipids are hydrolyzed by digestive enzymes and bile salts during *in vitro* human digestion. Owing to the presence of a double bond, cholesterol is also susceptible to oxidation when exposed to digestive enzymes and bile salts. Moreover, the activity of dietary antioxidant enzymes can be decreased during digestion and protein denaturation contributing to lipid and cholesterol oxidation. This study showed that the production of COPs could be increased during digestion, and is promoted by easy and quick cooking methods such as microwave heating, which induces lipid and cholesterol oxidation. Consequently, lipid digestibility correlated with increases in lipid peroxidation and COPs formation. Numerous studies have suggested that the formation of COPs and lipid oxidation in meat can be minimized by the application of low processing temperatures, that is through minimal processing, by the use of oxygen-proof packaging and a protective atmosphere as well as by low temperature and high free storage, by the dietary antioxidants to animals or antioxidants addition to meats (Hur *et al.*, 2007). A number of studies demonstrated that increased

consumption of oxidized fat is directly related to atherosclerosis and coronary disease. Therefore, there is clearly an urgent need for more research aimed at development of safer cooking methods, which would reduce consumption of injurious oxidized lipids and COPs.

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