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Development of microbial spoilage and lipid and protein oxidation in rabbit meat

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ABSTRACT

This experiment aimed to determine microbial spoilage and lipid and protein oxidation during aerobic refrigerated (4 °C) storage of rabbit meat. Forty male New Zealand white rabbits were slaughtered according to the Halal slaughter procedure. The hind limbs were used for microbial analysis while the *Longissimus lumborum* m. was used for determination of lipid and protein oxidation. Bacterial counts generally increased with aging time and the limit for fresh meat (10^8 cfu/g) was reached at d 7 postmortem. Significant differences in malondialdehyde content were observed after 3 d of storage. The thiol concentration significantly decreased with increase in aging time. The band intensities of myosin heavy chain and troponin T significantly reduced with increased refrigerated storage while actin remained relatively stable. This study thus proposes protein oxidation as a potential deteriorative change in refrigerated rabbit meat along with microbial spoilage and lipid oxidation.

1. Introduction

Due to its high digestibility, medium-low juiciness, little coarseness, odor and flavor, low-calories, lowest fatty feeling in the mouth and tenderness, rabbit meat is gaining preference by many meat consumers. Compared to red meats, rabbit meat has lower calories (on average 618 kJ/100 g fresh meat), fat (on average 6.8 g/100 g fresh meat), and cholesterol content (on average 53 mg/100 g fresh meat), iron (1.34 mg/100 g), sodium (47 mg/100 g) and energy (119 kcal/100 g) (Cavani, Petracci, Trocino, & Xiccato, 2009; Mačanga et al., 2011). Rabbit meat fits well the contemporary consumer demand for a low-fat meat with a high degree of unsaturated fatty acids and low cholesterol and sodium levels (Cavani et al., 2009; Hernández & Gondret, 2006).

Aging is one of the most critical factors that influence meat quality. During aging, the process of muscle conversion into meat is accompanied by quantitative changes in several metabolites. Consequently, meat becomes unfit for human consumption as it is considered to be spoiled. Spoilage of raw meat accounts greatly for major annual losses to meat processors and retailers (Nattress, Yost, & Baker, 2001). Bacteria levels between 10^7 and 10^9 cfu/cm^2 during refrigerated storage (Borch, Kant-Muemans, & Blixt, 1996) and TBARS values equal to or greater than 5 mg MDA/kg meat (Insausti et al., 2001) comprise the threshold for detecting fitness for human consumption. Owing to the assumption that lipid oxidation was, together with microbial spoilage, the only causes of food deterioration resulted in ignoring the fact that proteins are targets for reactive oxygen species (ROS) for several decades. The discovery that myofibril proteins are affected by ROS during meat maturation and storage (Martinaud et al., 1997) has prompted studies related to protein oxidation.

Oxidative reactions occur during storage and processing of meat, and meat products, and such processing steps such as mincing, cooking, and salt addition that promote the formation of ROS increase the susceptibility of products to oxidation. Protein oxidation induced by ROS can cause modification of backbones and side chains of proteins, which leads to structural changes at the levels of primary, secondary, and tertiary structures of proteins (Zhang, Xiao, & Ahn, 2013). These structural changes can induce conformational and functional modifications of proteins including protein solubility (Decker, Xiong, Calvert, Crum, & Blanchard, 1993; Nieto, Jongberg, Andersen, & Skibsted, 2013; Xiong, 2000), rehydration properties (Nieto et al., 2013; Xiong, 2000), protein fragmentation and aggregation (Decker et al., 1993; Promeyrat et al., 2011; Santé-Lhoutellier, Astruc, Marinova, Grève, & Gatellier, 2008), protein surface hydrophobicity (Santé-Lhoutellier, Astruc, Marinova, Grève, & Gatellier, 2008; Traore et al., 2012), viscosity, gelation and emulsification (Nieto et al., 2013; Xiong, 2000), and amino acid bioavailability (Santé-Lhoutellier, Astruc, Marinova, Grève, & Gatellier, 2008). It is also thought to negatively impact

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meat color (Filgueras et al., 2010; Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004a,2004b) and tenderness (Kim, Bødker, & Rosenvold, 2012; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007; Melody et al., 2004; Rowe et al., 2004a,2004b; Zakrys-Waliwander, O'Sullivan, O'Neill, & Kerry, 2012) and decrease water holding capacity (Decker et al., 1993; Melody et al., 2004).

Unlike red meats, there are a few studies about the shelf life and microbial quality of rabbit meat and the changes it may undergo during storage (Rodríguez-Calleja, García-López, Santos, & Otero, 2005; Rodríguez-Calleja, Isabel, García-López, Santos, & Andrées, 2006; Rodríguez-Calleja, Santos, Otero, & García-López, 2010; Sunki, Annapureddy, & Rao, 1978). Bobbitt (2002) studied the shelf life of rabbit carcasses stored at 4 °C and estimated a shelf life of only 3 d for boxed rabbit carcasses. However, in line with the study of Rodríguez-Calleja et al. (2005), a more recent study by Pereira and Malfeito-Ferreira (in press) has also reported a shelf life of 6–7 d under aerobic refrigerated storage. Lipid oxidation in rabbit meat is limited to the study of Nakyinsige et al. (2014) who reported 0.196 mg MDA/kg meat after 7 d of refrigerated storage at 4 °C while protein oxidation is limited to the studies of Gil et al. (2006) and Prates, Ribeiro, and Correia (2001) who reported degradation of myofibrillar proteins during rabbit meat aging. Thus this study sets out to determine microbial spoilage and lipid and protein oxidation of rabbit muscles during refrigerated storage.

2. Materials and methods

2.1. Ethical note

This study was conducted following the animal ethics guidelines of the Research Policy of Universiti Putra Malaysia.

2.2. Slaughter

A total of 40 male New Zealand white rabbits weighing between 1800 g and 2000 g were randomly chosen from thousands of others at a commercial farm (East Asia Rabbit Corporation) located in Semenyih, West Malaysia where they had been kept in cages and fed commercial rabbit pellet. The rabbits were transported for less than 1 h to the abattoir at the Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia where the slaughter was conducted. The animals were slaughtered according to Halal slaughter procedure as outlined in the Malaysian Standard MS1500: 2009 (Department of Standards Malaysia, 2009). The slaughter was performed by a licensed slaughter man by severing carotid artery, jugular vein, trachea and esophagus using a sharp knife.

2.3. Carcass sampling

After evisceration and carcass dressing, the left LL between the 6th and 8th lumbar vertebra was removed and divided into two, and snap frozen in liquid nitrogen before being stored at −80 °C. The first portion was assigned for subsequent determination of lipid and protein oxidation at d 0. The second portion was assigned for subsequent determination of TBARS at d 0. The right hind limbs and the fore arms were aseptically packed in stomacher bags and aerobically stored at 4 °C for microbial enumeration. The carcasses were then hung in the 4 °C chiller and the subsequent sampling was carried out at specific periods. The left LL muscle from the 9th to 12th lumbar vertebra was dissected into three portions at specific periods of 1, 3, 5 and 7 d post-mortem for subsequent analysis of TBARS. The right LL muscle that was divided into two parts from the 6th to 8th lumbar vertebra was used for the determination of ultimate pH. The portion from the 9th to 12th lumbar vertebra was further dissected at 3 specific periods, that is, 24 h and 7 and 14 d postmortem for determination of protein oxidation. Upon completion of each aging period, muscle chops of approximately 2.5 cm thickness were dissected from each specific muscle, labeled, vacuum packaged and stored at −80 °C until subsequent analyses.

2.4. Determination of pH

The pH of the meat was determined using a portable pH meter (Mettler Toledo, AG 8603, Switzerland) following the indirect method described by AMSA (2012). The samples were removed from −80 °C storage and manually pulverized in liquid nitrogen. Approximately 1.0 g of each crushed muscle sample was homogenized (Wiggen Hauser® D-500, Germany) for 30 s in 10 ml ice-cold deionized water in the presence of 5 mM sodium iodoacetate (Merck Schuchardt OHG, Germany) to prevent further glycolysis (specifically glyceraldehyde 3-phosphate dehydrogenase) or production of lactic acid (AMSA, 2012). The pH of the resultant homogenates was measured using the electrode attached to the pH meter.

2.5. Microbiological analysis

On each sampling day, 25 g of meat samples was aseptically weighed, transferred to a stomacher bag containing 225 ml of 0.1% of peptone water (Merck KGaA, Germany) and homogenized using a stomacher (Inter Science, France) for 120 s at room temperature. For microbial enumeration, 0.1 ml samples of serial dilutions (1:10 diluent, and peptone water) of homogenates were spread on the surface of dry media. Tenfold dilutions were spread plated in duplicate. Aerobic plate counts were enumerated on plate count agar (Merck KGaA, Germany) following 2 d of incubation at 30 °C (Harrigan, 1998; Rodríguez-Calleja et al., 2010). *Pseudomonas aeruginosa* numbers were determined, after 2 d of incubation at 25 °C (Rodríguez-Calleja et al., 2010) on Fluka® Analytical 70887-500G Cetrimide Agar (SIGMA-ALDRICH, Spain). *Escherichia coli* were enumerated after 24 h of incubation on Tryptone Soy Agar (CM0131, Oxoid, England) at 36 °C. *Brochothrix thermosphacta* was enumerated on streptomycin sulfate cycloheximide thallous acetate agar (STAA, Oxoid), supplemented with STAA Selective Supplement SR0151 E (Oxoid, England) following 18 h of incubation at 26 °C.

2.6. Lipid oxidation measurement

Lipid oxidation was measured as 2-thiobarbituric acid reactive substances (TBARS) using the QuantiChrom™ TBARS Assay Kit (DTBA-100, BioAssay Systems, USA) following the method of Nakyinsige et al. (2014). Concisely, samples were manually pulverized in liquid nitrogen. About 200 mg of the pulverized samples was mixed with 2 ml ice-cold phosphate buffered saline (PBS) and rapidly homogenized with an Ultra-Turrax T5FU (IKA-Labortechnik Staufen, Germany) for 20 s on ice. Two hundred microliters of homogenates was mixed with 200 μl of ice-cold 10% trichloroacetic acid (TCA) and incubated on crushed ice for 5 min. This was followed by centrifugation (Eppendorf Centrifuge, Mikro 22R Hettich, Germany) at 21,900 *g*, 4 °C for 5 min. Standards were prepared by mixing 15 μl of the 1.5 mM malondialdehyde (MDA) with 735 μl deionized water to obtain a final concentration of 30 μM MDA. Subsequently, 300, 180, 90 and 0 μl of 30 μM MDA were diluted with 0, 120, 210 and 300 μl of deionized water to generate the final 30, 18, 9 and 0 μM MDA as standards 1, 2, 3 and 4, respectively. Exactly 200 μl of thiobarbituric acid reagent was added to 200 μl of samples and standards and the mixture was incubated in a dry heating block at 100 °C for 60 min. Following equilibration to room temperature, 100 μl of standards and samples was loaded in duplicates into wells of a clear flat- bottom 96-well plate (Greiner Bio-One, Germany). Finally, optical density (OD) was determined at 535 nm (OD_{535}) using an auto UV Xenon flash lamp microplate reader (infinite M200, Tecan, Austria). After subtracting the OD of blank (standard 4) from all standard and sample values, a standard curve was obtained by plotting the ΔOD_{535} against standard concentrations. TBARS (μM MDA equivalent) concentration of

the samples was calculated according to the equation: [TBARS] = [(R_{sample} − R_{blank}) ÷ Slope (μM^{-1})] × n (Nakyinsige et al., 2014).

Where R_{sample} and R_{blank} are the OD_{535nm} of the sample and H_2O blank (STD_4) and n is the sample dilution factor (n = 3 for deproteinated samples).

2.7. Protein oxidation measurement

2.7.1. Extraction of myofibrillar proteins

Muscles were manually pulverized in liquid nitrogen. Myofibril proteins were isolated according to the method of Morzel, Gatellier, Sayd, Renerre, and Laville (2006) with some slight modifications. Approximately 2.5 g of frozen muscle was homogenized (Wiggen Hauser, D-500, Germany) for 30 s on ice in 25 ml of extraction buffer containing 150 mM NaCl, 25 mM KCl, 3 mM $MgCl_2$ and 4 mM EDTA at pH 6.5 to which protease inhibitor (CALBIOCHEM®, Cat # 55140, EMD Bioscience, Inc. Germany) had been added. The homogenate was filtered through 1.0 mm Polyethylene strainer to eliminate any remaining collagen. After filtration, the homogenate was incubated at 4 °C with shaking. This was followed by centrifugation at 2000 *g* for 15 min at 4 °C using an Avanti® J-26XPI centrifuge (BECKMAN COULTER®, USA). The pellet was washed twice with 25 ml of a 50 mM KCl solution at pH 6.4 and once with 25 ml of 20 mM phosphate buffer at pH 6. The pellet was finally resuspended in the same phosphate buffer and stored at −80 °C until analysis. The protein concentration of the samples was determined following the Bradford method using Protein Assay Kit II 500-0002 (Bio-Rad, USA). Bovine Serum Albumin (BSA) was used to prepare protein standards.

2.7.2. Determination of free thiol (SH) content

Thiol oxidation was measured according to Ellman's method of using 2, 2′-dithiobis (5-nitropyridine) DTNP (Winterbourn, 1990) with some modifications as outlined by Morzel et al. (2006). Stock solution containing 4 mg of myofibrillar proteins (Morzel et al., 2006) was dissolved in 3 ml of 100 mM phosphate buffer at pH 8 containing 8 M urea. About 30 μl of 10 mM DTNP (stock solution in ethanol) was added, followed by incubation for 1 h at room temperature. The absorbance at 386 nm was measured using a spectronic®20 GENESYS™ spectrophotometer (Spectronic instruments, USA) against a blank of buffer without protein. The absorbance of the blank was subtracted, and thiol concentration was calculated using an absorption coefficient of 14 mM^{-1} cm^{-1}. The final results were expressed as nanomoles of free thiol per milligram of protein.

2.7.3. SDS-PAGE

Myofibrillar proteins were mixed with sample buffer containing 30% (v/v) glycerol, 5% (v/v) mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris–HCl (pH 6.8) and 0.05% (w/v) bromophenol blue in a 1:1 ratio and incubated at 90 °C for 10 min. One dimensional SDS-PAGE was performed according to the method of Laemmli (1970) using polyacrylamide gels of 8 cm × 5.5 cm (length × width) and 0.8 mm thickness. Twelve percent resolving gels were prepared for actin and troponin T whereas 5% resolve gels were prepared for myosin heavy chain. The resolving gels were over-layered with 4% stacking gel solution and kept overnight at 4 °C in order to polymerize. A volume of 5 μl protein ladder (PageRuler™ Prestained Protein Ladder Plus; Cat No: SM 1811 from Fermentas Life Sciences, Canada) was loaded into the first well, while an equivalent of 20 μg proteins of each sample was loaded into the remaining wells. Proteins were separated in running buffer containing 0.025 M Tris base, 0.192 M glycine and 0.1 SDS at pH 8.3 under a constant voltage of 120 V and 400 mA for 90 min, in which the tracking dye reached the bottom of the gel. The gels were subsequently stained with 0.05% Coomassie blue staining solution for 60 min and destained with destaining solution for 30 min. The bands of myofibrillar proteins were visualized using a GS-800 Calibrated Imaging Densitometer (BIORAD, USA) (Fig. 1).

2.7.4. Western blotting

The fractionated proteins that were initially separated from the samples based on their molecular weight through gel electrophoresis were then transferred from the gel onto polyvinylidene difluoride (PVDF) membranes using Trans-Blot® SD semi-dry transfer system cell (BIORAD, USA). Myosin heavy chain was transferred at a constant amperage of 250 mA per gel and a voltage limit of 25 V for 135 min at

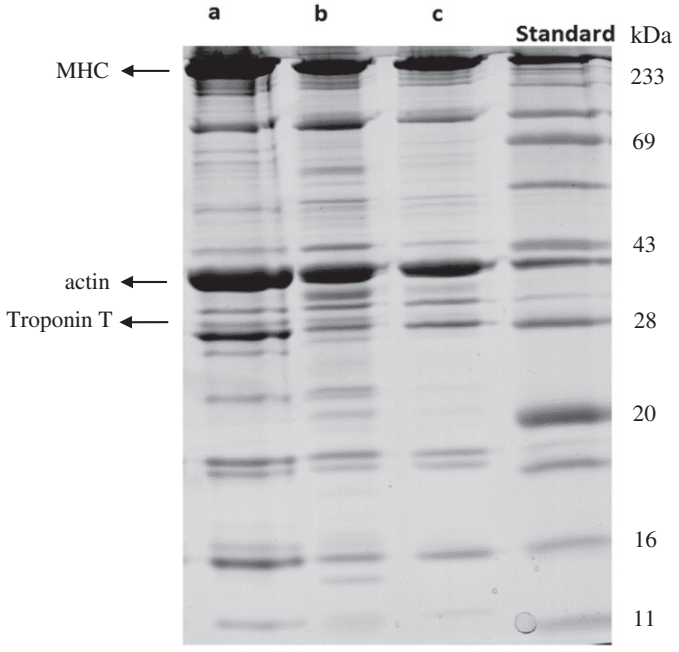

Fig. 1. Representative gel showing myofibrillar proteins in rabbit meat stored at 4 °C for 14 d. Lanes a–c: aging for 0, 7 and 14 d, respectively. Equal amount of protein (20 μg) of each sample was loaded and electrophoresed on a separate 12% SDS-PAGE under a constant voltage of 120 for about 90 min. The gels were then stained with Coomassie blue stain for 60 min and destained with destaining solution for 45 min. The bands of myofibrillar proteins were visualized using GS-800 Calibrated Imaging Densitometer.

Table 1
Effect of aging on the pH of rabbit LL muscle.

Parameter	Days postmortem			
	0	1	7	14
pH (unit)	6.530 ± 0.039[a]	6.188 ± 0.053[b]	6.038 ± 0.089[c]	6.031 ± 0.064[c]

[a,b,c]Means with different superscripts in the same row differed significantly at ($p < 0.05$). N = 40.

0 °C whereas actin and troponin T were transferred at the same amperage, voltage and time for 45 min. The membranes were immersed in a ready-to-use Ponceau S stain (aMReSCO®, Ohio) for 5 min to visualize the proteins of interest and to verify the electrophoretic transfer. The membranes were then washed with adequate deionized water, followed by one TBST buffer (100 mM Tris–HCl; 150 mM NaCl; 0.05% Tween 20) wash before being blocked with blocking buffer (5% BSA in TBST buffer) for 3 h at room temperature with constant shaking at 60 rpm on a wave shaker (MS Major Science, Taiwan). For myosin heavy chain, the membranes were incubated overnight with 1:500 dilution of primary antibody [Monoclonal Anti-Myosin (Skeletal, Fast), produced in mouse; Cat #. M4276 from Sigma-Aldrich, USA and Monoclonal Anti-Myosin (Skeletal, Slow), antibody produced in mouse; Cat # M842 from Sigma-Aldrich, USA in 3% BSA in TBST buffer. Anti-actin antibody produced in rabbit; Cat # A2066 from Sigma-Aldrich, USA and monoclonal anti-troponin T, antibody produced in mouse; Cat # T6277 from Sigma-Aldrich, USA were the primary antibodies used for actin and troponin T, respectively. Subsequently, the membranes were washed three times in TBST buffer (5 min incubation at room temperature, with constant shaking at 60 rpm on a wave shaker). The membranes were further incubated at room temperature in 1:10,000 dilution of secondary antibody [anti-mouse IgG (whole molecule)-peroxidase, antibody developed in rabbit; Cat # A9044 from Sigma-Aldrich, USA in 3% BSA in TBS-T buffer for 90 min]. This was followed by 3 times washing with TBST buffer. The blocked membranes were detected using a DAB substrate kit Code: E885 (DAB SUBSTRATE SYSTEM (aMReSCO®, Ohio)). Myosin heavy chain, actin and troponin T band intensities were measured by Quantity one software on a GS-800 Calibrated Imaging Densitometer (BIORAD, USA).

2.8. Statistical analysis

The experiment was of a completely randomized design. Data analysis was performed using the GLM procedure of Statistical Analysis System package (SAS) Version 9.1.3 software (Statistical Analysis System, SAS Institute Inc., Cary, NC, USA) and statistical significance was set at $p < 0.05$. Repeated measure design was used to analyze the progress of protein and lipid oxidation along the studied storage periods. A Duncan multiple range test was used to elucidate significantly different means. The correlation between lipid and protein oxidation was calculated by simple correlation and regression methods (SAS, 2005) assuming the following formula: $r_{X,Y} = Cov_{(X,Y)} / S_X S_y$, where: r(x,y) = correlation of the variables x and y; Cov(x, y) = covariance of the variables x and y; Sx = sample standard deviation of the random variable x; and Sy = sample standard deviation of the random variable y.

3. Results and discussion

3.1. Effect of aging on the development of microbial spoilage in rabbit meat

Table 1 shows the effect of aging on the development of microbial spoilage in rabbit meat during refrigerated storage at 4 °C. Bacterial counts generally increased with increase in aging time. Total aerobic counts were the highest, followed by *E. coli*, *P. aeruginosa* and lastly *B. thermosphacta*. The microbiological quality of meat is influenced by the physiological status of the animal at slaughter, the spread of contamination during slaughter and processing, the temperature and other conditions of storage (Koutsoumanis & Sofos, 2004). Bobbitt (2002) studied the shelf life of rabbit carcasses stored at 4 °C and estimated a shelf life of only 3 d for boxed rabbit carcasses. However, a more recent study by Pereira and Malfeito-Ferreira (in press) reported a shelf life of 6–7 d under aerobic refrigerated storage. Due to its specific composition, meat, particularly, rabbit meat with higher ultimate pH (Rodríguez-Calleja et al., 2005) comprises an excellent substrate for growth of spoilage bacteria. In the present study, rabbit meat also showed a relatively high ultimate pH with values above 6.0 (Table 2). These values are similar to those reported by Pereira and Malfeito-Ferreira (in press) (6.01–6.36) and by Rodríguez-Calleja et al. (2005) (6.26). The ICMSF (International Commission for Microbial Specifications in Food) recommends 10^6–10^7 cfu/cm^2 or g for fresh meat (ICMSF (International Commission for Microbial Specifications in Food), 1986). The total aerobic counts exceeded this limit at d 7. *E. coli* counts reached this value at d 7 while *P. aeruginosa* and *B. thermosphacta* did not reach this value. The shelf life of rabbit meat in the present study is higher than the 3 d reported by Bobbitt (2002) but similar to the 6–7 d reported by Pereira and Malfeito-Ferreira (in press) and Rodríguez-Calleja et al. (2005) at chilled aerobic storage.

3.2. Effect of aging on rabbit meat lipid oxidation

Lipid oxidation levels during the first seven days post-mortem are shown in Table 3. Generally, lipid oxidation increased ($p < 0.05$) with storage time. The MDA of the rabbit meat at d 0 and d 1 was not significantly different. The MDA content for d 3 and d 5 did not vary ($p > 0.05$) but was significantly higher than that of d 0 and d 1 and significantly lower than that of d 7. Lipid oxidation is an auto catalytic reaction, and thus the rate of oxidation increases as the reaction proceeds (Fernández, Pérez-Álverez, & Fernández-Lopez, 1997). Besides, the inherent antioxidant defense system of muscle remains active for a few days after animal death (Renerre, Poncet, Mercier, Gatellier, & Metro, 1999), explaining why statistical differences in TBARS values were only evident after 3 d of aging. These processes begin immediately after slaughter and their magnitude depends somewhat on the amount of pro-oxidants present in the system.

Autocatalytic oxidative processes of lipids constitute a major cause of reduced shelf life in meat, only second to microbial spoilage. Lipid oxidation has been implicated in the deterioration of flavor (Faustman, Sun, Mancini, & Suman, 2010; Fernández et al., 1997; Insausti et al., 2001; Jeremiah, 2001), formation of rancid odors (Fernández et al., 1997; Santé-Lhoutellier, Engel, & Gatellier, 2008), discoloration

Table 2
Effect of aging on microbial spoilage of rabbit meat.

Microorganism	Days postmortem			
	0	3	5	7
Total aerobic counts (log_{10} cfu/g)	4.688 ± 0.120[d]	6.177 ± 0.105[c]	6.783 ± 0.156[b]	7.831 ± 0.104[a]
Pseudomonas aeruginosa (log_{10} cfu/g)	3.436 ± 0.156[c]	3.751 ± 0.137[c]	4.484 ± 0.098[b]	5.579 ± 0.075[a]
Escherichia coli (log_{10} cfu/g)	2.781 ± 0.098[d]	3.280 ± 0.098[c]	5.099 ± 0.104[b]	6.729 ± 0.090[a]
Brochothrix thermosphacta (log_{10} cfu/g)	2.405 ± 0.055[c]	3.436 ± 0.063[b]	3.754 ± 0.025[b]	5.474 ± 0.564[a]

[a,b,c,d]Least square means with different superscripts in the same row indicate significant difference in the aging period at $p < 0.05$.
N = 40.

Table 3
Lipid oxidation of rabbit LL muscle along the storage period at 4 °C.

Parameter	Days post mortem				
	0	1	3	5	7
Malondialdehyde (mg/kg)	0.0141 ± 0.0013[c]	0.0263 ± 0.0046[c]	0.0697 ± 0110[b]	0.0782 ± 0.0141[b]	0.1527 ± 0.0098[a]

[a,b,c]Least square means with different superscripts in the same row indicate significant difference in the aging period at $p < 0.05$.
N = 40.

(Faustman et al., 2010; Juncher et al., 2001) and worst of all, production of potentially toxic compounds (Morrissey, Sheehy, Galvin, & Kerry, 1998; Richards, Modra, & Li, 2002) in meat. Lipid oxidation is induced by oxy- and/or lipid free radical generation and results in the formation of toxic compounds such as the malondialdehyde and cholesterol oxidation products (Morrissey et al., 1998). It has been reported that TBARS values equal to or greater than 5 mg MDA/kg meat comprise the threshold for the detection of off-odors and off-taste for humans (Insausti et al., 2001). However, this value was never reached in this study.

3.3. Effect of aging on loss of protein thiols

The quantification of protein thiols (the sulfhydryl group (SH) of a cysteine residue) together with the detection of protein disulfide cross-link formation by SDS-PAGE enables a detailed investigation of protein oxidation in food systems. Protein oxidation is associated with a decrease in sulfhydryl groups, which are converted into disulfides (Nieto et al., 2013; Soyer, Ozalp, Dalmıs, & Bilgin, 2010). The results for the effect of aging on free thiol content of rabbit LL muscle are presented in Table 4. Quantification of protein thiol groups showed that during 14 d of storage, the thiol concentration decreased from 34.7 to 24.3 nmol per mg protein. The protein thiols were found to significantly reduce as protein oxidation increased with increasing refrigerated storage. This finding is consistent with previous reports on chill storage of meat (Filgueras et al., 2010; Martinaud et al., 1997; Nieto et al., 2013; Zakrys-Waliwander et al., 2012).

Thiol groups of cysteine are oxidized with the formation of disulfide bridges. It was observed that thiols only oxidized to a certain level, which indicates that not all thiol-containing cysteine residues in the myofibrillar proteins exhibit similar reactivity. This observation is suggestive that some of the thiol groups are hidden inside the core of the protein, and therefore protected from oxidation. The decrease in thiols corresponds to the oxidation of accessible free thiol groups from cysteine residues located at the protein surface where as those cysteine residues located in the inner place could be protected against free radical attack (Sun, Cui, Zhao, Zhao, & Yang, 2011). Traore et al. (2012) indicated that oxidation levels do increase during storage in refrigerated conditions, but not greatly. Moreover, Levine, Berlett, Moskovitz, Mosoni, and Stadtman (1999) indicated that methionine and cysteine may have antioxidative capacity in proteins, and the abundance of cysteine residues in myosin makes it plausible that myosin-bound cysteines serve as radical scavengers when subjected to oxidative stress (Nieto et al., 2013). Post-mortem changes in the muscle include a decrease of the antioxidant defense system (Renerre et al., 1999) leading to an increase in the degree of lipid and protein oxidation (Martinaud et al., 1997; Renerre et al., 1999) under the action of free radicals.

Table 4
Effect of aging on free thiol (SH) content.

Parameter	Days postmortem		
	0	7	14
Thiol (nmol/mg protein)	34.692 ± 0.925[a]	29.231 ± 1.413[b]	24.250a ± 0.529[c]

[a,b,c]Means with different superscripts in the same row differed significantly at ($p < 0.05$).
N = 40.

3.4. Effect of aging on electrophoretic patterns of myosin heavy chain, actin and troponin T

Electrophoresis was performed in order to observe modifications in rabbit meat myofibrillar proteins during post-mortem aging. The SDS-PAGE patterns showed a decrease of bands corresponding to myosin heavy and light chains as post-mortem days increased (Fig. 1). The actin band was relatively more stable. The bands of troponin T also reduced with increase in aging period. The observed decrease in band intensity is indicative of protein oxidation in rabbit meat during refrigerated storage at 4 °C.

In their study on the influence of oxidation on bovine myofibrillar protein degradation, Xue, Huang, Huang, and Zhou (2013) showed that increased protein oxidation enhanced the degradation of myosin heavy chain (MHC) but had little influence on the degradation of actin. Martinaud et al. (1997) had earlier demonstrated that oxidation of myosin occurs naturally in meat during aging.

3.5. Effect of aging on degradation of rabbit meat myosin heavy chain, actin and troponin T

The intensities of MHC, actin and troponin T were quantified by measuring the reflective density (RD) of each detected band. As shown in Table 5, the RD of MHC and troponin T significantly reduced with increase in aging period ($p < 0.05$) whereas RD of actin did not significantly change during the 14 d storage at 4 °C.

Myosin heavy chain (MHC) protein is the major component of thick filament in sarcomeres and comprises approximately 35% of the total skeletal muscle protein (Lefaucheur, 2010). Electrophoretic studies by Morzel et al. (2006) and Stagsted, Bendixen, and Andersen (2004) showed that of all proteins, myosin was the most sensitive to oxidation and the first target in meat proteins, while lower molecular weight proteins seem to oxidize later. Decker et al. (1993) observed high molecular weight polymers produced by disulfide linkages mainly being derived from myosin and actin. Elsewhere, SDS-PAGE patterns have previously shown a decrease of band size and intensity of MHC after exposure to oxidant (Morzel et al., 2006; Xue et al., 2013). Ooizumi and Xiong (2004) indicated that the initial oxidation of chicken myofibrils induced changes in myosin, particularly intermolecular cross-linking of myosin heavy chain and modifications of thiol groups at the myosin ATPase active site. Highly oxidative conditions cause cross-linking, polymerization and aggregate formation in MHC through disulfide bonds, bityrosine and carbonyl (Morzel et al., 2006; Xue et al., 2013). Myofibrillar protein cross-linking in the form of binding and entrapping relating to the physical structures could affect meat tenderness (Huff-Lonergan & Lonergan, 2005). Furthermore, MHC isoforms have been reported to be significantly correlated with various aspects of meat quality including drip loss, juiciness, off flavor and tenderness attributes (Kanga et al., 2011). This is indicative that MHC oxidation may be an important influential factor of post-mortem meat tenderness.

Actin and actin-bundling proteins play an important role in muscle contraction. The current study showed that actin was not affected by postmortem storage at 4 °C. The bands remained relatively stable after d 14 (Fig. 1). Elsewhere, actin bands have also been found to be relatively stable even under oxidative conditions of μ-calpain (Xue et al., 2013) and chemical-induced oxidation (Morzel et al., 2006). Gil et al. (2006) indicated that actin is degraded very little or not at all during meat aging at 0–5 °C, even after 56 d and could only undergo substantial degradation at temperatures higher than 25 °C. This oxidative stability of actin may be attributable to inaccessibility of oxidation sites, which in myofibrillar suspensions may be masked by the interaction of actin with myosin chains.

Troponin T is the tropomyosin-binding component of the troponin complex that is involved in the calcium-dependent regulation of skeletal muscle contraction. The current study showed that the band size and intensity of troponin T significantly reduced during postmortem storage

Table 5
Effect of aging period on the degradation of myosin heavy chain, actin and troponin T in rabbit *Longissimus lumborum* muscle.

Myofibrillar protein	Days postmortem		
	0	7	14
Myosin heavy chain (reflective density/mm^2)	34.63 ± 2.10^a	30.09 ± 1.55^b	24.06 ± 2.56^c
Actin (reflective density/mm^2)	17.37 ± 1.07^a	17.12 ± 0.79^a	15.98 ± 1.00^{ab}
Troponin T (reflective density/mm^2)	12.87 ± 1.19^a	9.17 ± 1.43^b	5.73 ± 0.8^c

^{a,b,c}Least square means with different superscripts in the same row indicate significant difference in the aging period at $p < 0.05$.
N = 40.

at 4 °C (Table 5). In line with this observation, Penny and Dransfield (1979) and Martinaud et al. (1997) also reported that the degradation of troponin T progressed during the post-mortem storage of bovine muscles. Santé-Lhoutellier, Engel, Aubry, and Gatellier (2008) also reported that lamb storage affected the troponin T band after 7 d of storage. The appearance of a band in the zone of 30 kDa, attributed to the degradation of troponin T has been reported in aged rabbit meat (Gil et al., 2006; Prates et al., 2001). According to Harris, Huff-Lonergan, Lonergan, Jones, and Rankins (2001), the 30 kDa fragment derived from the parent troponin T can be a good indicator of meat aging and tenderization due to its close relationship with meat tenderness whereas Santé-Lhoutellier, Engel, Aubry, and Gatellier (2008) described it as a potential proteolysis index. Troponin T is present in the I-band regions of the intact myofibril, which undergo considerable breakage during muscle aging (Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995) and therefore its disruption, together with other I-band proteins titin and nebulin may contribute significantly to myofibril fragmentation and, thus, to tenderness (Huff-Lonergan, Parrish, & Robson, 1995).

In oxidative conditions, interaction of proteins with other biomolecules can also lead to cross-linking/polymerization, for example, aldehydic lipid oxidation products (malondialdehyde or 4-hydroxynonenal) can react with amino groups of proteins to form fluorescent aggregates known as lipofuscin or ceroid (Friguet, Stadtman, & Szewda, 1994; Grune, Jung, Merker, & Davies, 2004; Grune, Reinheckel, & Davies, 1997). Some studies have previously supported the timely coincidence of lipid and protein oxidation in meat systems (Estévez, Kylli, Puolanne, Kivikari, & Heinonen, 2008; Ventanas, Estévez, Tejeda, & Ruiz, 2006; Ventanas, Ventanas, Tovar, García, & Estévez, 2007). According to Estévez et al. (2008) and Vuorela, Salminen, Makela, and Kivikari (2005), the onset of lipid oxidation in meat systems seems to take place faster than the oxidative degradation of myofibrillar proteins, thus it is more likely that lipid-derived radicals and hydroperoxides promote protein oxidation than the vice versa. In fact, peroxyl radicals formed during lipid oxidation have been reported to abstract hydrogen atoms from protein molecules leading to a radical-mediated chain reaction similar to that of lipid oxidation (Stadtman & Levine, 2003). Soyer et al. (2010) reported good correlations between lipid and protein oxidation in chicken meat during frozen storage. However, in the present study, there was no correlation between lipid oxidation, as measured by TBARS, and protein oxidation, as measured by sulfhydryl content in rabbit LL muscles ($r = -0.28$, p value = 0.44) at d 7. Noteworthy, in the present study, as the MDA concentration increased, the thiol groups reduced, which indicates increase in protein oxidation. Despite the lack of correlation between protein and lipid oxidation, this observation can be attributed to the fact that both primary (hydroperoxides) and secondary (aldehydes) lipid oxidation products can react with proteins, causing the oxidation of proteins (Soyer et al., 2010).

4. Conclusion

The current results indicate that protein oxidation occurred during refrigerated storage of rabbit meat as suggested by loss of thiol groups and degradation of MHC and troponin T. This proposes protein oxidation as a potential deteriorative change in rabbit meat in addition to the commonly reported microbial spoilage and lipid oxidation.

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