Monitoring oxidation during the storage of pressure-treated cooked ham and impact on technological attributes

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ABSTRACT

High-pressure processing is a post-processing preservation method commonly used on meat products. However, it can affect the structural properties and the physico-chemical properties of the meat. The aim of this study was to compare the physical properties, lipid and protein oxidation of control and treated (500 MPa, 20 °C, 5 min) cooked ham during subsequent storage (21 days at 4 °C).

High pressure processing induced increase of hardness and syneresis after 7 days of storage. The redness (a*) was slightly affected by the high pressure treatment but not the lightness (L*) and the yellowness (b*). However, the fluctuation of color was not clearly visible. Evaluation of primary (conjugated dienes) and secondary (malondialdehyde MDA and thiobarbituric reactive substances TBA-RS) lipid oxidation products showed that pressure increases oxidation of lipids. Whereas, high pressure processing had no immediate effect on MDA and TBA-RS content, higher amount compared to control were observed during the refrigerated storage. This lipid oxidation could be due to the release of prooxidant iron from hemoproteins after the high pressure treatment. Finally, the determination of free and accessible thiols showed that the high pressure treatment leads to a protein oxidation.

1. Introduction

High-pressure processing is a non-thermal preservation/processing technique. It is used to extend the shelf life of food products without the use of preservatives or additives while minimizing impacts on the sensory and nutritional properties. HP treatment in the meat industry is mainly used as a post-packaging non-thermal decontamination technique (Sazonova et al., 2017). Indeed, the pressure levels applied in the meat industry range from 400 to 600 MPa, and short processing times at ambient temperature are generally used; these treatments result in the inactivation of the majority of pathogenic and spoilage microorganisms (Simonin et al., 2012).

In addition to the antimicrobial effects, high-pressure processing induces several physico-chemical changes. The extent and importance of these changes ultimately determine the commercial suitability of high-pressure processing of the meat product (Jofré and Serra, 2016). In particular, pressure affects the lipids and proteins. High pressure levels (>300 MPa) accelerate lipid oxidation in meat products, leading to the

formation of primary (conjugated dienes and hydroperoxides) and secondary (carbonyl compounds, ketones, alcohols and aldehydes) lipid oxidation products (Guyon et al., 2016). Lipid oxidation is an important parameter in determining stability, and it contributes to the nutritional properties and sensory qualities of high-pressure-treated products, influencing their acceptability. Lipid oxidation causes some detrimental effects, including decreased shelf life and nutritional value, increased off-flavours, and changes in sensory characteristics (colour and texture). Lipid oxidation also generates compounds that can exhibit harmful effects on human health due to carcinogenic and atherosclerotic effects, altering the composition of cell membranes, or reducing high-density lipoproteins (Vieira et al., 2017).

Muscle proteins are prone to oxidation by oxidized lipids, metal ions and other pro-oxidants generated during high-pressure processing. Oxidative reactions have been shown to induce a number of changes in proteins, such as modifications of the amino-acid side chains, formation of protein polymers resulting in a loss of solubility, increases in carbonyl groups and changes in the amino-acid composition (Xiong, 2000).

Finally, these changes can lead to a loss of essential amino acids and a decrease in protein digestibility, affecting the nutritional quality of the meat product. It has also been reported that the formation of carbonyl compounds due to protein oxidation can significantly alter the water retention, texture and colour of meat products (Estévez, 2011).

Cooked cured pork ham (hereafter, cooked ham) is one of the most consumed ready-to-eat meat products worldwide because of its high nutritional value, ease of use and appreciated sensory attributes (Benet et al., 2016). However, cooked ham is highly perishable due to its high water activity (approximately 0.98), its nearly neutral pH (approximately 6), and the absence of competing microbiota. Post-processing contamination by slicing or dicing is the greatest hazard. However, several studies have shown that high-pressure processing is a useful post-processing technique for inactivating food-borne pathogens and extending the shelf life of cooked ham (López-Caballero et al., 1999; Pietrzak et al., 2007; Vercammen et al., 2011). In addition, a previous enumeration of total mesophilic flora on Plate Count Agar medium according to standard NF EN ISO 4833 showed that a treatment at 500 MPa for 5 min at 20 °C maintained the mesophilic aerobic bacteria level below the detection limit (0.7 log CFU/g of ham) during 21 days of storage, while for untreated cooked ham, the content of mesophilic aerobic bacteria was 7.94 ± 0.08 log CFU/g of ham at 21 days of storage. Thus, a high-pressure treatment at 500 MPa for 5 min at 20 °C was effective in controlling the total flora and improve the shelf life of the cooked ham. However, it is known that the high-pressure treatment could induce physicochemical changes in the product and alter its sensory qualities. Thus, the aim of this study is to monitor the protein and lipid oxidation of high-pressure-treated "French superior cooked ham" during storage and to evaluate the likely consequences of this treatment on the technological attributes after the treatment and during the chilled storage.

2. Materials and methods

2.1. "Superior French cooked ham" preparation

Cooked ham was prepared from pork muscle (*Longissimus dorsi*) purchased from a local supermarket. Preparation was performed in the technology hall of Oniris (Nantes, France) according to a protocol already set up in a previous work (Rakotondramavo et al., 2019).

2.2. High-pressure treatment

High-pressure treatment was carried out in the technology hall of Oniris with a 3-L high-pressure pilot unit (ACB, Nantes, France). The cooked ham was previously diced (each side 1.5 cm) and vacuum packaged at 80 mbar in polyamide/polyethylene bags (La Bovida, Paris, France). The vacuum-packed samples were submitted to a high-pressure treatment at 500 MPa for 5 min at 20 °C.

2.3. Sample analyses

After the high-pressure treatment, the samples were stored in the dark at 4 °C for 21 days. The analyses were performed at 1, 7, 14 and 21 days after the high-pressure treatment. The physical analyses were performed with diced cooked ham, whereas for lipid, protein analyses and the determination of non-haem iron content, 50 g of ham was mixed (Waring blender) for 45 s; then aliquots were analysed.

2.4. Physical analyses

The syneresis and fluid loss during storage was determined for each sample by weighing the diced cooked ham that was vacuum packed and stored at 4 °C for 21 days. After pre-determined storage times (D+1, D+7, D+14 and D+21), the diced samples were unpacked, the meat was cleaned with absorbent paper and reweighed, and the weight loss (%) calculated. The results are expressed as the percentage of released water (exudate) relative to the initial sample weight. Four replicates were performed.

The surface colour of the diced cooked ham was determined using a Minolta CM 3500d (Konica Minolta, Tokyo, Japan) spectrophotometer. The colour is expressed in CIE L*a*b* values (lightness L*, redness a* and yellowness b*). For each condition, fifteen samples were analysed.

The hardness was determined using a TA.XT Plus texture analyser (Texture Technologies Corp. and Stable Micro Systems, Ltd., Hamilton, USA) equipped with a 50 kg load cell and a cylindrical probe 75 mm in diameter. Diced cooked ham samples were uniaxially compressed to 30% of their original height at a speed of 1 mm/s. Hardness corresponds to the peak force during the compression cycle. Fifteen replicates of each measurement were performed.

2.5. Non-haem iron content

The non-haem iron content was determined by the ferrozine method according to Villamonte et al. (2017). One gram of ground cooked ham was homogenized with 20 mL of citrate buffer (10 mM sodium citrate, pH 7, 140 mM NaCl) using an Ultra-Turrax homogenizer (T-25, IKA®, Staufen Im Breisgau, Germany) at 11000 rpm for 2×30 s. A dialysis was performed by placing a dialysis tube (cut-off value of 12 kDa) containing 4 mL of citrate buffer in the homogenate for 3.5 h.

One mL of dialysate was mixed with 10 μL of 10 mM ascorbic acid and 120 μL of 10 mM ferrozine. In parallel, a calibration solution, treated as the dialysate, containing iron sulfate at concentrations between 5 and 20 μM were tested. After 20 min, the absorbance at 562 nm was measured (Lambda 25, Perkin Elmer). The concentration of non-haem iron was determined using the standard curve prepared with the iron sulfate solutions. The results are expressed as μg of iron/g of cooked ham, and three replicates were performed for each measurement.

2.6. Lipid analysis

2.6.1. Lipid content and fatty acid composition analysis

Intramuscular lipids were extracted according to Folch et al. (1957). Ten grams of sample was mixed with 50 mL of solvent (chloroform/methanol; 2/1, v/v) using an Ultra-Turrax homogenizer (T-25, IKA®, Staufen Im Breisgau, Germany) at 11000 rpm for 1 min, and then the mixture was filtered through Whatman filter paper (N° 4). The pellet was washed with 50 mL of the same solvent and filtered again. The filtrates were combined, 5 mL of sodium chloride 0.73% was added, and the mixture was left to separate in a separating funnel. When two distinct phases were obtained, the lower phase containing the lipids and chloroform was extracted. The solvents were removed using a rotary evaporator under vacuum. The total lipid content was measured by weighing the residue after evaporation of the solvent (until constant weight). The fatty acid composition was determined according to the ISO/DIS 15304 standard. Three replicates were performed for each measurement.

2.6.2. Primary oxidation product: conjugated dienes

The conjugated dienes in the lipid extract were quantified according to the NF T60-223 (AFNOR, 2011) standard. Twenty milligrams of the lipid extract was mixed with 10 mL of isooctane. After homogenization, the absorbance of the solution was measured at 232 nm with a UV-visible spectrophotometer (Lambda 25, Perkin Elmer) with isooctane in the reference cell. The concentration of conjugated dienes was calculated using a molar extinction coefficient of 25200 M^{-1} cm^{-1}, and the results are expressed as μmol per g of sample. Three replicates were performed for each measurement.

2.6.3. Secondary oxidation products: malondialdehyde (MDA) and thiobarbituric reactive substances (TBA-RS)

The extent of lipid oxidation is commonly measured by the TBA test. 2-Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) and other aldehydes to form a (MDA-TBA) pink-coloured complex with

fluorescent properties. The intensity of the colour, and consequently the amount of complex produced, is proportional to the MDA content and allows the oxidation state of the fatty substances to be quantified. Then, the MDA concentration can be evaluated by spectrophotometric analysis of the thiobarbituric reactive substances (TBA-RS), and the results are expressed as μmol MDA equivalents. However, the TBA reagent can react not only with MDA but also with many other compounds, interfering in the TBA test and leading to overestimation of the MDA content. Thus, high-performance liquid chromatography (HPLC) techniques offer better specificity and sensitivity in MDA determinations as measurements are made after separation of the (MDA-TBA) complex.

The TBA-RS values were determined according to Villamonte et al. (2017) with slight modifications. Four grams of sample was homogenized in an ice bath at 11000 rpm for 2×35 s with 40 μL of 4.5 mM BHT in ethanol, 80 μL of 100 mM EDTA and 10 mL of 0.3 M trichloroacetic acid using an Ultra-Turrax homogenizer in an ice bath. The homogenate was vortexed for 1 min, sonicated in an ice bath for 5 min and centrifuged at 4000 g for 10 min. The supernatant was incubated (1:1, v/v) with 55 mM TBA reagent at 70 °C for 20 min and then cooled under running cold water. The absorbance was recorded from 450 to 600 nm against a blank prepared with the reagents. The absorbance at 532 nm was corrected by subtracting the absorbance at 575 nm to limit overestimation related to the turbidity of the reaction medium. The TBA-RS concentrations were calculated from a standard curve using hydrolysed 1,1,3,3-tetramethoxypropane (TMP) as a precursor of MDA. The results are expressed in nanomole of MDA equivalent/g of cooked ham. Three replicates were performed for each measurement.

MDA was also quantified by high-performance liquid chromatography (HPLC) (Kenmogne-Domguia et al., 2012). Samples were prepared following the procedure described for the quantification of TBA-RS. Twenty microliters of the reaction mixture were injected into a u-HPLC system equipped with a C18 column (Symmetry, 150×4.6 mm, 5 μm) and a diode array detector (DAD) at a wavelength of 532 nm. The MDA-$(TBA)_2$ complex was eluted with a mobile phase composed of NaH_2PO_4/Na_2HPO_4 buffer (50 mM, pH 6.8)/methanol/acetonitrile (72/17/11; v/v/v) at a flow rate of 1 mL/min. MDA was quantified using an external calibration curve prepared with TMP. The results are expressed in nanomole of MDA/g of cooked ham. Three replicates were performed for each measurement.

2.7. Protein analysis

2.7.1. Protein extraction

One gram of ground cooked ham was mixed with 9 mL of 0.04 M potassium phosphate buffer at pH 6 and containing potassium chloride (0.6 M). The mixture was homogenized with an Ultra-Turrax homogenizer for 2×30 s at 11000 rpm. The protein homogenate was then filtered through gauze in order to eliminate the connective tissue and the residual lipids. The protein concentration in the homogenate was determined using the Biuret method. This protein extract will be used for the different protein analyses: quantification of total carbonyls and quantification of free and accessible thiols. Three replicates were performed for each sample.

2.7.2. Quantification of total carbonyls

The quantification of carbonyl compounds was determined by reacting them with DNPH (2,4-dinitrophenyl hydrazine) to form a yellow precipitate with an absorbance peak at 370 nm according to the protocol of Levine et al. (1994). The method was set up and described in a previous work (Rakotondramavo et al., 2019).

2.7.3. Quantification of the free and accessible thiols

The thiol function (SH sulfhydryl group) can be quantified by reaction with 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent) yielding a measurable yellow compound at 412 nm. The protocol was inspired by Guyon et al. (2018). Both free thiols and accessible thiols were

determined.

For this, after the protein extraction, an aliquot was mixed with urea to determine the accessible thiols. The urea is a chaotropic agent that disrupt aggregates and hydrogen bonds and thus allows an increased accessibility of buried SH groups. Then, another aliquot was mixed with the extraction buffer to determine the free thiol. The method was described and set up in a previous work (Rakotondramavo et al., 2019).

2.8. Statistical analysis

The effects of the factors (high-pressure treatment and storage time) were analysed by two-way analysis of variance (ANOVA) using Statgraphics Centurion XVII software (Statpoint Technologies, Warrenton, USA). This procedure displays various tests to determine the factors that have a statistically significant effect on the variables (syneresis, a*, b*, L*, non-haem iron content, MDA and TBA content, total carbonyls content, free and accessible thiols). It also tests if there are significant interactions between factors. The F tests allows to identify significant factors. The significance of effects and interactions were verified using Tukey's test with a confidence level of 5%. The correlation between the lipid and protein oxidation parameters and the physical parameters was evaluated by Pearson's correlation test, and $p < 0.05$ represents a statistically significant difference.

3. Results and discussion

3.1. Effects of high-pressure processing on the lipid oxidation of cooked ham

3.1.1. Fatty acid composition and non-haem iron content

Compounds involved in lipid oxidation include unsaturated fatty acids (substrates) and chemical species such as iron that can initiate the reaction. The intramuscular lipid content, fatty acid composition and non-haem content were measured. The lipid contents of untreated and pressurized cooked ham were respectively 2.47 ± 0.28 g/100g of ham and 2.20 ± 0.20 g/100 g of ham. These contents are lower than the average lipid content of the superior French cooked ham 4.28 g/100 g (Ciqual, 2017). This is due to the low initial lipid content of *longissimus dorsi*, muscle which is used, but also to the trimming step of the intramuscular fat made during the preparation of the meat before cooked ham manufacturing.

Table 1 shows the fatty acid composition of cooked ham. Cooked ham contains approximately 38% saturated fatty acids (SFA), 49% monounsaturated fatty acids (MUFA) and 14% polyunsaturated fatty acids (PUFA). The most abundant fatty acid in cooked ham was oleic acid (C18:1 n-9). There were no significant differences in the percentages of

Table 1

Fatty acid composition of cooked and pressurized cooked ham.

Fatty acid (%)	Cooked ham	Pressurized cooked ham
C14:0	1.3 ± 0.1	1.2 ± 0.1
C16:0	23.1 ± 0.4	23.1 ± 0.6
C18:0	12.4 ± 0.2	12.5 ± 0.3
C20:0	0.2 ± 0.0	0.2 ± 0.0
C16:1	3.3 ± 0.1	3.2 ± 0.1
C18:1 (n-9)	40.3 ± 1.2	40.3 ± 1.6
C18:1 (n-7)	4.3 ± 0.0	4.2 ± 0.1
C20:1	0.7 ± 0.0	0.7 ± 0.0
C18:2 (n-6)	9.4 ± 0.4	9.4 ± 0.7
C18:3 (n-6)	0.1 ± 0.0	0.1 ± 0.0
C18:3 (n-3)	0.3 ± 0.0	0.3 ± 0.0
C20:2	0.3 ± 0.1	0.3 ± 0.0
C20:3	0.4 ± 0.0	0.4 ± 0.0
C20:4	2.4 ± 0.2	2.4 ± 0.1
Saturated Fatty Acid (SFA)	37.7 ± 0.5	37.8 ± 1.0
Mono Unsaturated Fatty Acid (MUFA)	48.7 ± 1.2	48.6 ± 1.8
Poly Unsaturated Fatty Acid (PUFA)	13.6 ± 0.8	13.6 ± 0.8
n-6/n-3	16.7 ± 1.3	18.4 ± 1.2

the individual or total fatty acids between the non-treated and pressure-treated samples.

The non-haem iron content of cooked ham and pressurized cooked ham during refrigerated storage of are presented in Fig. 1. The initial free iron content in untreated cooked ham was 0.81 ± 0.20 μg/g. This content remained almost constant during storage. At day 1, high-pressure treatment had no effect on the non-haem iron content in cooked ham ($p > 0.05$). However, a significant increase was observed after 7 days of storage: from 0.83 ± 0.08 μg/g of ham at day 1–1.76 ± 0.23 μg/g of ham at day 7 ($+53\%$), and then the content remained stable until day 21. Thus, the two factors, pressure and storage time, influenced the non-haem iron content. The main effect of each factor and the interaction of the two factors on the non-haem content were presented in Table 2. The increase in the non-haem iron content with HP treatment during the first week of refrigerated storage may be due to the opening of the haem by pressure. This behaviour was in agreement with a study on high-pressure-treated chevon meat that showed an increase in the non-haem iron content during storage (Jalarama Reddy, Jayathilakan, Chauhan, Pandey & Radhakrishna, 2015).

3.1.2. Conjugated dienes, TBA-RS and MDA contents following high-pressure processing

The effects of HP treatment on lipid oxidation in cooked ham, as indicated by the contents of primary oxidation products (conjugated dienes) and secondary oxidation products (MDA and TBA-RS), during 21 days of storage at 4 °C are shown in Table 3.

The initial conjugated diene content in cooked ham was 0.17 ± 0.03 μmol/g of ham. A significant increase was observed in the first 7 days of storage (increase of 48%). No significant change was noticed during the subsequent 21 days of storage. High-pressure processing had no significant effect on the initial conjugated diene content in cooked ham. However, the conjugated diene content in high-pressure-treated cooked ham increased gradually until day 21. At the end of the storage period (21 days), the high-pressure-treated cooked ham presented a higher

Fig. 1. Non-haem iron contents (μg/g of ham) during 21 days of storage. *Different letters* indicate significant differences ($p < 0.05$).

Table 2
Two ways results of variance analysis for non-haem iron content according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21).

Source	Sum of squares	DoF	Middle square	F-test	Probability p
MAIN EFFECTS					
A: HP treatment	1.65373	1	1.65373	69.21	0.0000
B: Storage duration	1.65886	3	0.552955	23.14	0.0000
INTERACTIONS					
AB	0.628048	3	0.209349	8.76	0.0011
RESIDUE	0.38232	16	0.023895		
TOTAL (CORRECTED)	4.32296	23			

conjugated diene content (0.57 ± 0.02 μmol/g of ham) than that of untreated cooked ham (0.37 ± 0.03 μmol/g of ham). Conjugated dienes, which are primary oxidation products, are relatively unstable, and they can be rapidly converted into secondary oxidation products (Guyon et al., 2016).

The general trends observed for MDA and TBA-RS contents during storage were similar for both untreated and high-pressure-treated samples. However, the TBA-RS content tended to be slightly higher than the MDA content. The most widely used method for determining MDA is the spectrophotometric determination of the pink colour caused by the MDA-thiobarbituric acid (MDA-TBA) complex produced by the reaction with 2-thiobarbituric acid (TBA). However, this method is criticized for its lack of specificity (or selectivity) and its high inaccuracy since TBA reacts not only with MDA but also with many other compounds that could interfere with the TBA assay, resulting in a considerable overestimation of the MDA content. Thus, the higher values obtained from the TBA tests can be attributed to several other lipid oxidation products. In fact, other than MDA, the oxidation of unsaturated fatty acids can generate a variety of volatile compounds. For example, in cooked pork products, octanal, nonanal and 2-undecenal are oxidation products of oleic acid (C18:1) (Frankel, 2005), which is the major fatty acid (approximately 40% of the total fatty acids) in the cooked ham used in this study; hexanal, 3-nonenal and 2,4-decadienal are the main volatile oxidation products of linoleic acid (C18: 2, ω-6, approximately 9.4% of the total fatty acids). All these compounds can react with TBA. Thus, a more accurate MDA assay by u-HPLC has been used.

The MDA content of untreated cooked ham was stable during storage. High pressure processing increased the production of MDA until day 14 when the content was the highest (MDA: 2.99 ± 0.62 nmol/g of ham). Then, the content decreased significantly by day 21 (MDA: 1.67 ± 0.09 nmol/g of ham).

Thus, high pressure does not appear to affect lipid oxidation immediately after the treatment, whereas it seems to favour lipid oxidation during prolonged storage. In fact, lipid oxidation in the treated cooked ham was enhanced during storage. Thus, the two factors, pressure and storage time, influenced the TBA-RS and MDA content. The main effect of each factor and the interaction of the two factors on the TBA-RS and MDA content were presented in Table 4. Two mechanisms have been suggested to explain the changes induced by pressure treatment. First, the increase in the rate of lipid oxidation has been suggested to be caused by the release of iron ions from haemoproteins during high-pressure processing (Carlez et al., 1995). It is generally accepted that iron in some forms promotes the oxidation of meat lipids. Of the various forms of iron, non-haem iron has been reported to play a major role in accelerating lipid oxidation. It has been shown that simple salts (iron sulfate, ferrous ammonium sulfate, and ferric chloride) added to a cooked meat model system acted as prooxidants of lipid oxidation (Min et al., 2010). Conversely, Vieira et al. (2017) compared the effects of haem pigments and non-haem iron on lipid oxidation in different types of meat and concluded that haem pigment induced a greater accumulation of TBA-RS than did non-haem iron. Thus, the relative contributions of haem and non-haem iron to lipid oxidation in meat and meat products are still widely debated. In addition, some authors have suggested that high-pressure processing can promote lipid oxidation by disrupting the cytoplasmic membranes of lipids. Huang, He, Li, Li & Wu (2012) confirmed that phospholipids are the main lipids altered at 500 MPa (20 min, 20 °C). Membrane disruption facilitates contact between unsaturated lipids from the membrane and catalysts such as haem, non-haem iron and other metal cations and thus may contribute to lipid oxidation (Bolumar et al., 2014). In this study, lipid oxidation seems to be related to non-haem iron because a simultaneous increase in the contents of non-haem iron and secondary oxidation products (MDA and TBA-RS) between D+1 and D+7 was observed in the pressure-treated cooked ham. Furthermore, in the untreated cooked ham, the MDA, TBA-RS and non-haem iron contents all remained stable.
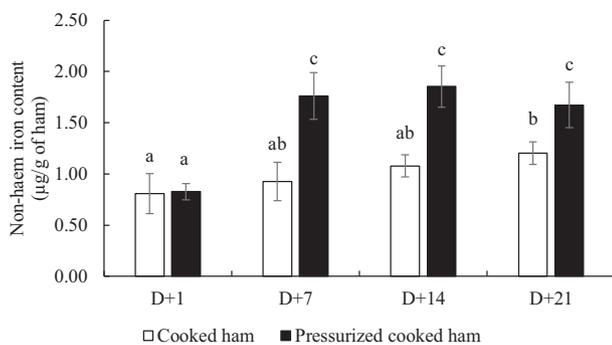
Table 3

Conjugated diene contents (μmol/g of ham), TBA-RS contents (nmol/g of ham), MDA contents (nmol/g of ham) and total carbonyls (μmol/g of protein) in untreated cooked ham and pressurized cooked ham during 21 days of storage. Mean ± confidence interval. For each variable (conjugated diene, TBA-RS, MDA and total carbonyls), *different letters* in the line (storage time) and column (untreated cooked ham and pressurized cooked ham) indicate significant differences at p ≤ .05.

Storage time	Conjugated diene (μmol/g of ham)		TBARS (nmol/g of ham)		MDA (nmol/g of ham)		Total carbonyls (μmol/g of protein)	
	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham
D+1	0.17 ± 0.03 *a*	0.20 ± 0.02 *a*	1.17 ± 0.20 *b*	0.86 ± 0.03 *a*	1.01 ± 0.03 *b*	0.73 ± 0.03 *a*	1.40 ± 0.18 *a*	1.38 ± 0.18 *a*
D+7	0.33 ± 0.03 *b*	0.40 ± 0.03 *c*	1.56 ± 0.25 *bc*	2.87 ± 0.38 *d*	1.22 ± 0.15 *c*	2.27 ± 0.02 *e*	1.62 ± 0.14 *a*	1.60 ± 0.40 *a*
D+14	0.39 ± 0.03 *bc*	0.42 ± 0.03 *c*	1.61 ± 0.21 *c*	3.67 ± 0.78 *d*	1.07 ± 0.04 *bc*	2.99 ± 0.62 *e*	1.60 ± 0.22 *a*	1.53 ± 0.16 *a*
D+21	0.37 ± 0.03 *bc*	0.57 ± 0.02 *d*	1.67 ± 0.09 *c*	1.93 ± 0.37 *c*	1.22 ± 0.02 *c*	1.70 ± 0.05 *d*	1.40 ± 0.29 *a*	1.38 ± 0.26 *a*

3.2. Effect of HP treatment on protein oxidation in cooked ham

The oxidation of proteins results in the production of various oxidized derivatives. The main oxidation sites of proteins are the side chains of the amino acids, and potential modifications include thiol oxidation, aromatic hydroxylation, and the formation of carbonyl groups (Zhang et al., 2013). The generation of carbonyls is the most common protein oxidation pathway. At day 1, the amounts of carbonyl compounds in untreated and treated cooked ham were respectively 1.48 ± 0.20 μmol/g of protein and 1.38 ± 0.18 μmol/g of protein and those values remain stable during the refrigerated storage (Table 3). Then, neither HP treatment nor storage time significantly affected the carbonyl content in cooked ham.

To evaluate another aspect of protein oxidation, the accessible and free thiol content was assessed (Fig. 2). Indeed, a decrease in the content of free SH groups is often linked to protein oxidation. In addition, the accessible thiols were also determined to evaluate the reversibility of the thiol oxidation. Accessible thiols include SH groups exposed at the surface or buried in the protein structure. Here, urea was used as a chaotropic agent that disrupt aggregates and hydrogen bonds and thus allows an increased accessibility of SH groups.

The thiol content of untreated cooked ham is shown in Fig. 2A. The initial accessible thiol content (159.26 ± 10.78 μmol/g of protein) of untreated cooked ham was significantly higher than the free thiol content (123.53 ± 11.95 μmol/g of protein). This indicates that some thiol oxidation had already taken place during the manufacturing process, during brining, cooking, and dicing, for example. Then, the accessible thiol content decreased significantly (by 29%) from day 1 to day 7 and by 20% from day 7 to day 14. A decrease in the free thiol content was also observed between day 1 and day 7. At day 7 and day 14, the free and accessible thiol contents were similar. This decrease in the thiol content could be attributed to reactive oxygen species (ROS) directly attacking the thiol groups of the cysteine residues in meat proteins and converting them to disulfide bonds and other thiol oxidation products, such as sulfenic, sulfinic and sulfonic acids and thiosulfinates (Rysman et al., 2016). In addition, the decrease in the content of accessible thiols implies that the thiol oxidation was an irreversible reaction. At day 21, the content of accessible thiols remained unchanged relative to that on day 14, whereas the content of free thiols had further decreased. Thus, thiol oxidation continues until day 21. However, the thiol reactions that were occurring at day 21 were reversible because the amount of accessible thiols did not decrease. Indeed, according to Rysman et al. (2014) thiol oxidation is complex and may lead to the formation of multiple oxidation products, of which disulfides and sulfenic acid are formed reversibly.

The accessible thiol contents in pressure-treated cooked ham (Fig. 2B) are significantly higher than in untreated cooked ham ($p < 0.05$). Throughout chilled storage, the concentration of accessible thiols in treated cooked ham gradually decreased, but the concentration remained higher than those of untreated cooked ham. This shows that high-pressure processing induced protein denaturation in the cooked ham, leading to increase accessibility of the SH groups, by inducing protein denaturation. The conformational changes in the proteins and especially myofibrillar proteins caused by high-pressure processing expose the SH groups buried within the protein structure making them accessible. These results are consistent with those of Chapleau et al. (2002) on myofibrillar proteins.

At day 1, the content of accessible thiols in treated cooked ham was significantly higher than that of free thiols (188.06 ± 9.11 μmol/g of protein and 156.80 ± 5.32 μmol/g of protein, respectively). These levels are higher than those in untreated cooked ham. This means that the HP treatment induced a thiol reaction. At day 7, the concentration of accessible thiols in the pressure-treated cooked ham remained the same as that at day 1, while the free thiol content decreased sharply until reaching the same level as that in the untreated cooked ham (96.34 ± 4.21 μmol/g of protein and 100.62 ± 8.64 μmol/g of protein, respectively). Unlike untreated cooked ham, the thiol reaction that occurred between day 1 and day 7 in the treated cooked ham was reversible. After day 7, the contents of accessible and free thiols in untreated cooked ham decreased until day 21, but the accessible thiol content remained higher than the free thiol content. These results show the co-occurrence of reversible and irreversible thiol reactions in high-pressure-treated cooked ham, unlike in untreated cooked ham in which irreversible reactions are favoured. This shows the disorganization of the protein structure induced by high-pressure processing continues during chilled storage.

3.3. Effect of HP treatment on the physical parameters of cooked ham

The effects of high-pressure processing on the physical properties of cooked ham during 21 days of storage are shown in Table 5. The main effect of each factors (pressure and storage time) and the interaction of

Table 4

Two ways results of variance analysis for TBA-RS and MDA. The ANOVA table breaks down the variability of variable according to the different factors. The values of the probabilities test the statistical significance of each of the factors ($p < 0.05$).

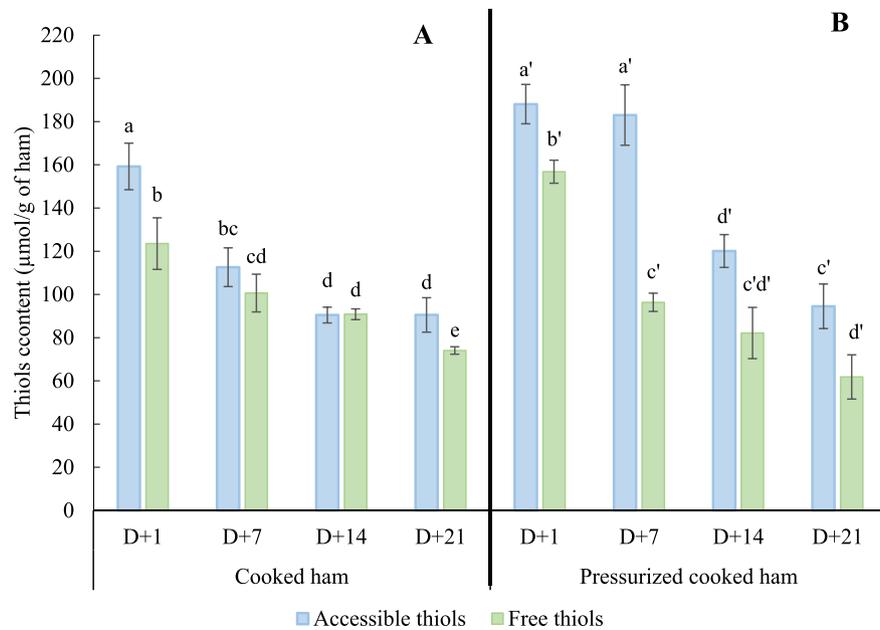| Source	Sum of squares	DoF	Middle square	F-test	Probability p
TBA-RS content according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21).					
MAIN EFFECTS					
A: HP treatment	4.1417	1	4.1417	40.96	0.0000
B: Storage duration	8.58991	3	2.8633	28.32	0.0000
INTERACTIONS					
AB	5.04601	3	1.682	16.63	0.0000
RESIDUE	1.61793	16	0.101121		
TOTAL (CORRECTED)	19.3956	23			
MDA content according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21)					
MAIN EFFECTS					
A: HP treatment	3.7525	1	3.7525	94.02	0.0000
B: Storage duration	4.39425	3	1.46475	36.70	0.0000
INTERACTIONS					
AB	3.85115	3	1.28372	32.16	0.0000
RESIDUE	0.6386	16	0.0399125		
TOTAL (CORRECTED)	12.6365	23			

Fig. 2. Amount of free and accessible thiols in untreated cooked ham (A) and pressure-treated cooked ham (B). *Different letters* indicate significant differences ($p < 0.05$).

the two factors on the physical properties (syneresis, a*, b*, L* and hardness) were presented in Table 6.

Syneresis is associated with liquid exudation during chilled storage, which is one of the main problems with vacuum-packed meat products because consumers intuitively recognize it as a lack of meat juiciness. Fluid accumulation can also favour the growth of microorganisms. The water released from the cooked ham at day 1 was $6.11 \pm 2.05\%$, and this value remains stable during refrigerated storage. High-pressure processing had no significant effect ($p > 0.05$). on the syneresis of cooked ham at day 1 However, the syneresis increased significantly (+35%) to D+7 and continued to increase but not significantly until D+21. Pietrzak et al. (2007) observed a significant increase in syneresis in cooked ham treated at 600 MPa (20 °C, 10 min). In contrast, López-Caballero et al. (1999) did not observe a difference in syneresis between untreated cooked ham and cooked ham treated at 400 MPa (7 °C, 20 min). Thus, water retention of cooked ham is influenced by the level of pressure and the temperature used during the treatment.

The values of a* of the samples varied between 1.89 ± 0.41 and 4.34 ± 0.33, those of b* between 7.17 ± 0.42 and 8.32 ± 0.38 and those of L* between 59.64 ± 1.80 and 62.81 ± 0.91. These values are significantly different from the colour parameter values of cooked ham reported in the literature. Specifically, the values of a* found in this study are lower than

those found in the literature, whereas the values of b* and L* are greater than those found in the literature (Pietrzak et al., 2007; Pancrazio et al., 2015; Pingen et al., 2016). These differences could be due to the lower concentration of myoglobin in the muscle used in this study and differences in the nitrite concentration in the brine.

Neither the lightness (L*) nor the yellowness (b*) of the cooked ham were affected by HP treatment, and these parameters did not change during refrigerated storage for either untreated or pressure-treated cooked ham.

The redness of the cooked ham did not change significantly until day 7 of storage. The value of a* increased significantly (+28%) from day 14 compared to day 1 and then remained constant until day 21 of storage. This increase corresponds to the development of the typical pink colour of cooked ham. Nitrite addition during the curing step of cooked ham manufacturing causes the characteristic pink colour associated with cured products. Added nitrite binds with deoxymyoglobin, causing a rapid reduction of the bound nitrite to nitric oxide (NO) myoglobin (nitrosomyoglobin). This pigment is not stable before the cooking step. Denaturation of the NO-myoglobin during cooking exposes the centrally located porphyrin ring, resulting in final cured pigment, nitrosylhaemochrome, due to the interaction between ferrous iron and NO. This pigment generated by cooking becomes increasingly stable during

Table 5
Physical parameters of untreated cooked ham and pressurized cooked ham during 21 days of storage. Mean $\pm$ confidence interval. For each variable (syneresis, a*, b*, L* and hardness), *different letters* in the line (storage time) and column (untreated cooked ham and pressurized cooked ham) indicate significant differences at $p \leq .05$.

Storage time	Syneresis (%)		a*		b*		L*		Hardness (N)	
	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham	Cooked ham	Pressurized cooked ham
D+1	6.1 ± 2.1 a	7.0 ± 0.7 a	2.9 ± 0.5 b	1.9 ± 0.4 a	8.1 ± 0.4 b	8.3 ± 0.4 b	61.6 ± 0.8 a	62.6 ± 0.7 a	32.5 ± 3.2 a	38.5 ± 3.8 ab
D+7	6.5 ± 2.1 a	10.9 ± 1.8 b	3.8 ± 0.6 bc	3.3 ± 0.7 bc	7.2 ± 0.6 a	8.0 ± 0.7 b	62.7 ± 0.9 a	62.6 ± 1.4 a	36.6 ± 4.0 a	45.5 ± 3.4 b
D+14	8.7 ± 2.0 a	15.9 ± 3.7 b	4.7 ± 0.2 c	4.0 ± 0.5 c	7.6 ± 0.4 ab	7.2 ± 0.8 ab	61.3 ± 1.4 a	59.6 ± 1.8 a	34.4 ± 3.3 a	43.4 ± 2.9 b
D+21	9.1 ± 2.8 a	14.5 ± 2.6 b	4.3 ± 0.3 c	4.2 ± 0.8 c	7.3 ± 0.4 ab	7.2 ± 0.7 ab	62.8 ± 0.9 a	62.2 ± 1.4 a	34.5 ± 4.0 a	44.3 ± 2.2 b

Table 6
Two ways results of variance analysis for syneresis, a*, b*, L* and hardness.

Source	Sum of squares	DoF	Middle square	F-test	Probabilité p
Syneresis according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21)					
MAIN EFFECTS					
A: HP treatment	121.341	1	121.341	29.62	0.0001
B: Storage duration	130.063	3	43.3544	10.58	0.0004
INTERACTIONS					
AB	31.2087	3	10.4029	2.54	0.0931
RESIDUE	65.5384	16	4.09615		
TOTAL (CORRECTED)	348.151	23			
a* according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21)					
MAIN EFFECTS					
A: HP treatment	19.3739	1	19.3739	21.62	0.0000
B: Storage duration	42.3078	3	14.1026	15.74	0.0000
INTERACTIONS					
AB	12.6956	3	4.23186	4.72	0.0041
RESIDUE	83.331	93	0.896032		
TOTAL (CORRECTED)	160.094	100			
b* according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21)					
MAIN EFFECTS					
A: HP treatment	19.0241	1	19.0241	22.68	0.0000
B: Storage duration	29.4609	3	9.82029	11.71	0.0000
INTERACTIONS					
AB	9.74314	3	3.24771	3.87	0.0117
RESIDUE	78.0073	93	0.838788		
TOTAL (CORRECTED)	132.748	100			
L* according to the high pressure treatment (cooked ham and pressurized cooked ham) and the storage duration (D+1, D+7, D+14 and D+21)					
MAIN EFFECTS					
A: HP treatment	2.35456	1	2.35456	0.59	0.4433
B: Storage duration	64.5097	3	21.5032	5.41	0.0018
INTERACTIONS					
AB	26.1325	3	8.71082	2.19	0.0941
RESIDUE	369.387	93	3.97191		
TOTAL (CORRECTED)	457.851	100			

storage, corresponding to an increase in the red index (a*) during storage (Mattos et al., 2003).

At day 1 of storage, the redness (a*) of the pressure-treated cooked ham is lower (1.89 ± 0.41) than that of untreated cooked ham (2.91 ± 0.45). The reduction in the a* value after high-pressure treatment can be attributed to the oxidation of ferrous myoglobin to ferric metmyoglobin (Carlez et al., 1995) or to the denaturation of myofibrillar proteins (Goutefongea et al., 1995). This second hypothesis is preferred in the case of cooked ham because metmyoglobin is stabilized by nitrite, forming nitrosomyoglobin after cooking. This decrease in a* after HP treatment has also been observed for other cured meat products (Andrés et al., 2006; Tanzi et al., 2004). The redness of pressure-treated cooked ham increased during storage to reach the same value as that of untreated cooked ham at D+21.

However, despite these slight changes in the value of a*, the overall colour of high-pressure-treated cooked ham remained visually identical to that of the untreated cooked ham. Indeed, in theory, in cured meat products, nitrosomyoglobin, which is responsible for the pink colour of cooked ham, is quite resistant to HP treatment (Jofré and Serra, 2016), so the slight fluctuations in colour are not detectable.

The hardness results obtained for cooked ham and high-pressure treated cooked ham are presented in Table 5. High-pressure processing had no significant effect on the hardness of cooked ham on day 1. However, a difference appeared as storage progressed. While the hardness of the untreated sample remained stable for 21 days, that of the treated sample increased significantly between day 1 and day 7 and then stabilized until day 21. High-pressure processing therefore has no immediate effect on the hardness of cooked ham but tends to increase the hardness during early refrigerated storage. These hardness results may be correlated with the syneresis results. The increase in the hardness of pressure-treated cooked ham coincides with the increase in water loss.

The water loss caused by HP treatment would therefore lead to a firmer product during refrigerated storage.

3.4. Relationship between the technological properties and oxidation of cooked ham calculated within HP treatments and refrigerated storage times

The correlation coefficients obtained by pooling information within the storage times, HP treatment, technological properties and oxidation indicators are presented in Table 7.

The redness of the cooked ham was not correlated with high-pressure processing but was significantly correlated with storage time ($r = 0.412$, $p < 0.05$). The redness is also negatively correlated with the free thiol content ($r = -0.453$, $p < 0.05$). The hardness was highly correlated with pressure treatment ($r = 0.741$, $p < 0.05$) but not with storage time. The conformational changes in the proteins caused by high-pressure treatment are associated with changes in the strengths of the intra- and intermolecular interactions, which include protein-water interactions. According to the Le Chatelier principle, this change leads to a more compact structure. In addition, these conformational changes may cause the denaturation of myofibrillary and sarcoplasmic proteins, resulting in the aggregation of their subunits (Marcos et al., 2010); the structural rearrangements could be responsible for the increase in hardness (Clariana et al., 2011). Hardness was also correlated with syneresis ($r = 0.503$, $p < 0.05$). Indeed, the increase in meat hardness after HP treatment can be accompanied by a significant decrease in water retention by the meat (Duranton et al., 2012).

Hardness and syneresis were both correlated with lipid oxidation indicators (conjugated dienes and MDA) and negatively correlated with the free thiol content, which is an indicator of protein oxidation. Indeed, pressure-induced denaturation would lead to the formation of aggregates, most likely generated through intermolecular disulfide bridges.

Table 7

Pearson's coefficients of the physical and chemical variables studied. * indicates a significant effect at $p < 0.05$.

Variables	Storage	Pressure treatment	a*	Hardness	Syneresis	Non-heme iron	Carbonyls	Free thiols	Accessible thiols	Conjugated dienes	MDA
Storage	1										
Pressure level	0,000	1									
a*	0,412*	-0,191	1								
Hardness	0,115	0,741*	0,048	1							
Syneresis	0,562*	0,590*	0,114	0,503*	1						
Non-heme iron	0,510*	0,619*	0,225	0,656*	0,822*	1					
Carbonyls	-0,279	0,057	0,246	-0,028	-0,117	0,065	1				
Free thiols	-0,882*	0,035	-0,453*	-0,159	-0,523*	-0,605*	0,044	1			
Accessible thiols	-0,818*	0,425*	-0,404	0,209	-0,216	-0,091	0,287	0,770*	1		
Conjugated dienes	0,820*	0,351	0,330	0,415*	0,716*	0,736*	0,008	-0,848*	-0,583*	1	
MDA	0,301	0,544*	0,186	0,566*	0,743*	0,868*	0,110	-0,495*	-0,034	0,526*	1

During the cooking step, heat induced the unfolding of native proteins, which can lead to the formation of protein aggregates. First, cysteine residues (sulfhydryl groups) located in the heavy myosin head groups and disulfide bonds that are not accessible in the native conformation can become accessible and may react to form intermolecular cross links. Then, noncovalent aggregation occurs, and the aggregation process may continue during the step-by-step cooking process until a gel is formed (Gravelle et al., 2016). The accessibility of the disulfide bridges is rather limited in heat-denatured proteins, which explains why the accessible thiol content was lower in untreated cooked ham, as mentioned above (Fig. 2). When HP treatment is applied, the cross links and disulfide bridges established during the cooking step can be disrupted (Visschers and de Jongh, 2005). As seen in the results of the accessible thiol contents (Fig. 2), HP increased the amount of reactive SH groups, which contributed to the formation of the gel network in the meat. Such rearrangements cause a gradual coarsening of the gel structure, which may lead to exudation of fluid. This could explain the correlation between syneresis and HP treatment as well as the free thiol content. In addition, the accessibility of reactive SH groups after high-pressure processing could lead to covalent aggregation (irreversible formation of products), which gives rise to a more compact gel. Consistent with this, a positive correlation between hardness and syneresis was observed ($r = 0.503$, $p < 0.05$). In addition, MDA can react with the sulfhydryl groups of proteins, altering their functionality (Papastergiadis et al., 2012), which could influence the texture and syneresis of the meat product.

The accessible and free thiol contents were both negatively correlated with storage time ($r = -0.818$ and $r = -0.882$, respectively, $p < 0.05$). However, only the accessible thiol content was correlated with pressure treatment ($r = 0.412$, $p < 0.05$). Thus, high-pressure processing leads to increased accessibility of reactive thiol groups, and these groups are released during storage, leading to the reversible and irreversible formation of thiol oxidation products. The non-haem iron content was strongly correlated with the MDA and conjugated diene contents. This indicates that, in this study, lipid oxidation was induced by non-haem iron released during HP treatment.

4. Conclusion

High-pressure treatment (500 MPa-20 °C-5 min) of "superior French cooked ham" induced protein oxidation by the reaction of sulfhydryl groups rather than by the production of carbonyl compounds. Indeed, protein thiols undergo reversible and irreversible oxidation during chilled storage. Furthermore, high-pressure treatment had no significant effect on lipid oxidation immediately after treatment, but it did during refrigerated storage. This lipid oxidation seems strongly related to the iron released during storage and initiated by HP treatment. These oxidative reactions lead to significant physical changes, especially in hardness and syneresis during storage, which may be discriminating criteria for the consumer. However, the effect of high-pressure treatment

on the colour parameters of cooked ham was so slight. The effects of high pressure are strongly dependent on the studied matrix, but these results can elucidate the oxidation phenomena occurring in cooked meat products subjected to high pressure. However, further studies, in particular on the contribution of haem iron and fatty acids to the oxidation phenomena, should be undertaken. A study of the effects of high-pressure treatment on denaturation and aggregation could explain the increases in hardness and syneresis.

Declarations

Author contribution statement

Anja Rakotondramavo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Lucie Ribourg, Claire Guyon: Performed the experiments; Analyzed and interpreted the data.

Anne Meynier, Marie de Lamballerie, Laurence Pottier: Conceived and designed the experiments; Analyzed and interpreted the data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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