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Implication in nitrosation, nitrosylation and oxidation

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Abstract

Nitrite, added to cured meat for its bacteriological and technological properties, is implicated in the formation of nitroso compounds (NOCs), such as nitrosylheme, nitrosamines and nitrosothiols, suspected to have a potential impact on human health. The mechanisms involved in NOC formation are studied in regard with the dose-response relationship of added nitrite and its interaction with ascorbate on NOC formation in a cured and cooked meat model. The impact of a second cooking stage on nitrosation was evaluated. The addition of nitrite in the cured and cooked model promoted heme iron nitrosylation and S-nitrosation but not N-nitrosation. Nitrite reduced lipid oxidation without an additional ascorbate effect. The second cooking sharply increased the nitrosamine content while the presence of ascorbate

considerably lowered their levels and protected nitrosothiols from degradation. This study gives new insights on the chemical reactivity of NOCs in a cured meat model.

Key words

Nitrite, ham, nitrosamine, nitrosylheme, nitrosothiol

1. Introduction

Nitrite and nitrate are used in cured meat processing to prevent the growth of harmful bacteria and develop the color and flavor of products. The ability of nitrite to limit the growth of bacteria, especially *Clostridium botulinum* strains which produce toxins was formerly demonstrated (Skibsted, 2011). Nitrite also promotes the conservation of cured meat thanks to its antioxidant capacity (Berardo, De Maere, Stavropoulou, Rysman, Leroy, & De Smet, 2016).

Nevertheless, the use of nitrite in food has been debated for several years. The risk of nitrite comes from its ability to form nitroso compounds (NOCs), some of which are mutagenic. Indeed, nitrite can react with secondary amines (N-nitrosation) to form nitrosamines (De Mey, De Maere, Paelinck, & Fraeye, 2017). Nitrosamines exist in two forms, volatile and non-volatile, depending on their boiling point. Nitrosamines are considered as volatile when they can be distilled from strong solutions of salts or caustic soda with a minimum recovery of 80%. The involvement of nitrosamines in carcinogenesis is due to their enzymatic degradation into reactive diazo compounds, which can cause damage to DNA (Tricker & Preussmann, 1991). Nitrite can also react with heme iron to form nitrosylheme

(nitrosylation), responsible for the characteristic color of cured meat (Honikel, 2008), and with free thiol groups (S-nitrosation) to form nitrosothiols (Kuhnle & Bingham, 2007). Both nitrosylheme and nitrosothiols are known to release NO and can act as nitrosating agents during digestion (Kuhnle & Bingham, 2007). Nitrate is not dangerous at the levels found in meat products but it can be reduced into nitrite by certain microorganisms and in the human body, in the oral cavity (van Maanen, van Geel, & Kleinjans, 1996).

Lipid oxidation in meat can lead to the formation of mutagenic aldehydes (Sottero, Leonarduzzi, Testa, Gargiulo, Poli, & Biasi, 2019). Several epidemiological studies have suggested that excessive consumption of cooked meat and cured meat is associated with an increased risk of colorectal cancer (Larsson & Wolk, 2006). To limit oxidation and the formation of nitrosamines, antioxidants such as sodium ascorbate are usually added to cooked cured meat. Nonetheless, ascorbate has an ambivalent role, depending on the physico-chemical environment. By reducing heme iron (Richards, 2013) and nitrous acid into nitric oxide (Honikel, 2008; Skibsted, 2011), ascorbate contributes to heme iron nitrosylation. In some conditions, ascorbate can also act as a pro-oxidant (Amaral, da Silva, & da Silva Lannes, 2018).

Recent eating habits encourage consumers towards new cured meat consumption patterns and people are consuming more and more ready-to-eat meals. These new practices have to be taken into account when studying the chemical reactivity of nitrite. For instance, heating pizzas will necessarily lead to a second cooking for pizza toppings such as ham, bacon bits, and chorizo. The temperature applied is in most cases higher than 150°C. Yet such conditions favor the formation of mutagenic compounds and especially nitrosamines in cured meat (Herrmann, Duedahl-Olesen, & Granby, 2015a).

To prevent the risk of nitroso-compound production in cured meat, it is essential to investigate the mechanisms involved in their formation. As a precursor of the formation of

NOCs, nitrite is suspected to be involved in carcinogenesis (Santarelli et al., 2010). Nevertheless, the dose effect of nitrite, combined with the addition of ascorbate and their interaction during cured meat processing are little documented (Herrmann, Granby, & Duedahl-Olesen, 2015b; Higuero, Moreno, Lavado, Vidal-Aragon, & Cava, 2020) and, to our knowledge, the second cooking was never investigated.

The aim of this study is thus to get a better understanding of the reactions and interactions of the compounds involved in NOC formation in cured products. To this end, a model of cooked cured meat was studied to assess the influence of composition, *i.e.* sodium nitrite doses and ascorbate addition, and chemical interactions. Heat treatment at high temperature mimicking a second cooking of products was performed to evaluate the chemical reactivity of nitrite and ascorbate, through the formation of non-volatile nitrosamines and nitrosothiols.

2. Material and Methods

2.1. Reagents

All the reagents, *i.e.* ferrozine, ascorbate, acetone, hydrochloric acid, thiobarbituric acid, ethanol, tempol, o-phenylenediamine (OPDA), and the Griess reagent kit for nitrite and nitrate assays (ref : 23479-1KT-F) were purchased from Sigma Aldrich (Saint Louis, USA). Vivaspin® 2 system (PES membrane with a cut-off of 5 kDa) (ref: VS0212) and the syringe filters with 0.22 µm regenerated cellulose membranes (ref: 17761) were purchased from Sartorius (Göttingen, Germany).

2.2. Preparation of cured meat samples

2.2.1. Cured and cooked meat model preparation

The experiment was carried out on thirty-three porcine shoulders from Piétrain x (Landrace x Large White) pigs (100 kg weight), slaughtered in a commercial slaughterhouse after electric stunning. The shoulders were defatted, derinded, denerved and deboned. To prevent inter-animal and muscle variability, muscles of all animals were pooled and frozen at -20°C prior to meat preparation.

This model is based on the DCNO model (Dark Cooked with Nitrite and Oxidized), reported by Santarelli et al. (2010), by its composition in heme iron and nitrite. After thawing, meat was cut into large pieces (approximately 50mm), mixed with brine under vacuum, and then churned for one night before cooking at a heat of 68.5°C for 48 min. Brine (containing natural NaCl salt and sodium nitrite salt at various concentration, and dextrose) was mixed with meat at 100 g/kg under vacuum conditions. The exact composition of the brine is displayed in Supplementary Material 1. Different brine compositions in sodium nitrite were used, leading to final rates of 40 ppm – the sufficient rate to add color and aroma (Alahakoon, Jayasena, Ramachandra, & Jo, 2015), 80 ppm – the short-term goal of the profession, and 120 ppm – the maximum authorized by the French Code of Practice (Code des usages de la charcuterie, de la salaison et des conserves de viande. *IFIP Ed.* Institut du Porc ; 2017). A control without added nitrite was also performed. Sodium ascorbate was added at 300 mg per kg of meat, and a control without ascorbate was performed with 120 ppm of sodium nitrite. In the following article, 0ppm+A; 40ppm+A; 80ppm+A; 120ppm+A correspond respectively to 0, 40, 80 and 120 ppm of sodium nitrite added in the presence of ascorbate. Samples 120ppm-A correspond to 120 ppm of sodium nitrite added without ascorbate. Samples were stored under vacuum at 4°C until analysis or a second cooking stage.

2.2.2. Second cooking of the cured and cooked meat model

125 The samples described in previous section were heated in an oven at 220°C for 6 min.
126 Moreover, to be more representative of domestic habits, samples treated with 120 ppm nitrite
127 with or without ascorbate were heated under two other temperature duration conditions:
128 180°C for 7 min and 260°C for 4 min. The experimental design of the cooking processes is
129 displayed in Supplementary Material 2.
130 The second cooking was performed on a thin water film (3-5 millimeters) to limit
131 dehydration and mimic what happens with the wet constituents of a pizza for instance. The
132 samples from the second cooking were frozen and stored at - 80°C until analysis.
133
134 2.3. Biochemical characterization
135 2.3.1. Determination of nitrite and nitrate content
136
137 Nitrite and nitrate were extracted using the method of Ionescu, Zara, Aprodu, Vasile, and
138 Carac (2006) , with slight modifications. Samples (2 g) were homogenized with a Polytron
139 PT 2100 (KINEMATICA AG, Switzerland) for 30 seconds in 10 mL of water and 2.4 mL of
140 2% NaOH. Then, 10 mL of water and 420 µL of 7.4% HCl, was added to samples to reach
141 pH 8. The samples were homogenized with a vortex and incubated at 50°C for 15 minutes.
142 Finally, the samples were deproteinized by centrifugation with a Vivaspin® 2 system with a 5
143 kDa cut-off threshold (16°C, 900g for 75 min with an SL 40R centrifuge from Thermo
144 Scientific, Waltham, USA). The supernatants were eliminated and the filtrates were used for
145 assays.
146 Nitrite and nitrate ion contents were then determined in the filtrates using the Griess reaction
147 with a Sigma-Aldrich colorimetric assay kit. Absorbance was measured on a microplate at
148 540 nm on a MULTISKAN SPECTRUM spectrophotometer from Thermo Scientific
149 (Waltham, USA). Residual nitrite and nitrate were expressed in mg/ kg of wet matter (ppm).

150

151 2.3.2. Determination nitrosothiol content

152

153 The filtrates (1 mL) prepared in section 2.3.1 were saturated with $HgCl_2$ (10 mg) to
154 specifically cleave the S-NO bonds of nitrosothiols (Gaston, 1999). The samples were filtered
155 on syringe filters with 0.22 µm regenerated cellulose membrane. Nitrite ion content was
156 measured using the Griess reaction with the Sigma-Aldrich colorimetric assay kit and the
157 difference between this measure and the level of nitrite ions initially present in the sample
158 (section 2.3.1) gave the nitrosothiol content (Gaston, 1999). Nitrosothiols were expressed in
159 mg S-NO/ kg of wet matter (ppm). A considerable loss of water was observed during the
160 second cooking stage (around 60%, data not shown). To prevent any artefact due to drip loss,
161 the nitrosothiol values were expressed in relation to dry matter in mg/ kg of dry matter.

162

163 2.3.3. Determination of non-volatile nitrosamine content

164

165 The filtrates prepared in section 2.3.1 were irradiated with a UV lamp (model LF 215.S, 254
166 nm, 2x15 W) from Uvitec (Cambridge, England) with a sample distance with source of d =
167 2cm. UV irradiation cleaves the S-NO bonds of nitrosothiols and the N-NO bonds of
168 nitrosamines and nitrosamides (Breider & von Gunten, 2017), giving a mixture of nitrite and
169 nitrate. C-nitroso and O-nitroso containing compounds, oximes, nitramines, and N-oxide are
170 stable under UV light and do not interfere in the assay (Breider & von Gunten, 2017).
171 Nitrosylheme iron does not interfere in the assays because of the withdrawal of nitroso-
172 myoglobin during Vivaspin centrifugation. If released, nitrosyl iron (Fe-NO) is rapidly
173 transformed, in oxidative medium, into more stable $Fe[(OH)ONOO^-]^{2-}$ (Fotiou, Fotiou, &
174 Deliconstantinos, 2009) with a UV resistant O-NO bond.

175 Irradiation kinetics were sampled at 1, 15, 30, 60 and 120 minutes of exposure. The content
176 of both nitrite and nitrate was measured using the Griess reaction. The concentration of [NO^{2-}
177 + NO^{3-}] was plotted versus the inverse of time and extrapolation to 1/t = 0, obtained by fitting
178 a second-degree polynomial function, giving the maximum rate of nitrite + nitrate released.
179 This measure gave the sum of nitrosothiol, non-volatile nitrosamine and nitrosamide content.
180 The subtraction of nitrosothiol content, obtained as described in section 2.3.2, gave the non-
181 volatile nitrosamine and the nitrosamide contents (Apparent Total N-Nitroso Compounds,
182 ATNC). Nevertheless, nitrosamides decompose rapidly and, contrary to nitrosamines, they do
183 not accumulate in food products, so their level is generally not detectable (Chow, Dhaliwal,
184 & Polo, 1984). Finally, non-volatile nitrosamines were expressed in mg N-NO/ kg of wet
185 matter (ppm) while, regarding nitrosothiols, the nitrosamine content after the second cooking
186 stage were expressed in relation to dry matter.
187
188 This method is described in more detail in Bonifacie et al. (submitted in MethodsX).
189
190 2.3.4. Determination of ascorbate content
191
192 Total ascorbate content and the two forms of ascorbate, *i.e.* oxidized and reduced, were
193 evaluated in the samples according to Vislisel, Schafer and Buettner (2007). The samples (0.8
194 g) were extracted in 4 mL of sodium acetate (2M, pH 5.5). After agitation and
195 homogenization, 1 mL of extract was mixed with a ratio of 1/5 in 250 µM EDTA prepared in
196 ethanol/H_2O (90/10) (v/v). The samples were centrifuged at 4°C, 4000 rpm for 15 min. 100
197 µL of Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy) was added in 100 µL of the
198 samples. After agitation at 22°C at 600 rpm for 10 min, the ascorbate was oxidized into
199 dehydroascorbate (DHA). O-phenylenediamine (OPDA) (42µL) was added and reacted with

DHA to form the fluorescent OPDA-DHA complex. In the absence of Tempol, only the naturally oxidized ascorbate present in the medium can react with OPDA. The difference between the two measurements gave the level of reduced ascorbate. The fluorescence of the OPDA-DHA complex was measured in 96-well black polystyrene microplates with a Jasco FP-8300 spectrofluorometer, (Jasco, Oklahoma City, USA) (λex/em = 345/425nm). The results were expressed in ppm.

2.3.5. Determination of non-heme iron content

The non-heme iron content and the Fe^{2+}/Fe^{3+} ratio were measured by the ferrozine assay, according to Stolze, Dadak, Liu and Nohl (1996) and adapted to meat samples. Non-heme iron was separated from heme iron by a centrifugation method using a Vivaspin® 2 system at 18°C, 4000 rpm for 75 min. The absorbance of the ferrozine-iron complex was measured at 562 nm on a multiskan spectrum from Thermo Scientific (Waltham, USA) and a standard calibration curve is used to calculate the non-heme iron content in samples (Supplementary Material 3). The results were expressed in µM.

2.3.6. Evaluation of heme iron nitrosylation

The selective extraction of nitrosylheme was carried out by adding acetone to the sample, at a ratio of 4:1 (v/w) with 300 µL of water. After homogenization with a polytron, the samples were filtered on folded filters (ref: 1202-150, Whatman). The level of nitrosylheme was evaluated by measuring specific absorbance at 540 nm and using an absorption coefficient of 11,3 mM^{-1} cm^{-1} (Hornsey, 1956) on a Jacso V-770 spectrometer (Jasco, Oklahoma City, USA). In parallel, the total heme iron was estimated in the form of acid haematin by

225 extraction in acidic acetone. After homogenization, the samples were filtered and the total
226 heme iron was estimated by measuring specific absorbance at 512 nm with an absorption
227 coefficient of 9.52 mM^{-1} cm^{-1} (Hornsey, 1956). The nitrosylation of heme iron was expressed
228 as the percentage of nitrosylheme to total heme iron.
229
230 2.3.7. Lipid oxidation measurement
231
232 Lipid extraction was performed using the procedure of Folch, Lees and Sloane-Stanley
233 (1957) with slight modifications. To do this, the samples (1g) were ground in 4 mL of water
234 with a polytron. Ethanol-dichloromethane (1/2 (v/v)) was added to the sample extract at a
235 ratio of 3 (solvent):1 (sample) and the mixture was shaken vigorously. After evaporation the
236 concentration of lipids was calculated by weighing the dry residues. In parallel, lipid
237 oxidation was measured by the thiobarbituric acid reactive substances (TBARS) method
238 according to Mercier, Gatellier, Viau, Remignon, and Renerre (1998). The results of lipid
239 oxidation were then expressed as mg of malondialdehyde (MDA) per g of lipids.
240
241 2.3.8. Protein oxidation measurement
242
243 Protein oxidation was evaluated by the free thiols content measurement, with a modification
244 of Ellman's method using 2,2'-dithiobis(5-nitropyridine) (DTNP) (Winterbourn, 1990). The
245 adduct products of DTNP on free thiols were evaluated by their absorbance at 386 nm on a
246 Jacso V-770 spectrometer (Jasco, Oklahoma City, USA) using an absorption coefficient of 14
247 $mM^{-1}.cm^{-1}$. In parallel, the total protein content was evaluated by the Biuret method. The
248 results were expressed as nanomoles of DTNP bound per milligram of protein.
249

250 2.3.9. Dry matter

251

252 Dry matter was evaluated by heating samples at 105°C for 16 hours and by weighing the dry
253 residues (da Silva, Ezequiel, Diesel, Lunedo, & Cleef, 2011).

254

255 2.4. Statistical analysis

256

257 The statistical analyses were carried out with STATISTICA software (version 13.3) from
258 TIBCO Software Inc. (Palo Alto, USA). The values for each experimental condition were
259 reported as the mean ± standard error of the mean (SEM) of six independent repetitions. The
260 effect of nitrite and ascorbate treatment in the cooked model were assessed independently by
261 a one-way analysis of variance (ANOVA): (0ppm+A ; 40ppm+A ; 80ppm+A ; 120ppm+A)
262 and (120ppm+A ; 120ppm-A) respectively. The effect of second cooking conditions and
263 sodium nitrite doses were tested together by ANOVA (0ppm+A ; 40ppm+A ; 80ppm+A ;
264 120ppm+A at 220°C for 6 minutes). The effect of second cooking conditions and ascorbate
265 addition were tested together by ANOVA (120ppm+A ; 120ppm-A at 180°C for 7 minutes ;
266 220°C for 6 minutes; 260°C for 4 minutes). All ANOVA were performed using a confidence
267 level of 5%, and the *post hoc* test used was the Tukey test. A principal component analysis
268 (PCA) was also performed with STATISTICA software using centered reduced data.

269

270 3. Results and Discussion

271

272 In the cured and cooked meat model (section 3.1.) all the nitroso compounds and their
273 precursors, catalysts, and inhibitors were determined in relationship with sodium nitrite doses
274 and ascorbate. In the second cooking model (section 3.2.), only nitrosothiols and nitrosamines

were evaluated with regard to the doses of sodium nitrite added and the presence or not of ascorbate. At high temperature, as used in the second cooking, nitrosylheme can lead to various products, like hemochrome, hemichrome, oxo ferryl species and biliverdin, that are impossible to evaluate because of their visible absorbance very close to that of nitrosylheme. Moreover, iron or nitrosyl iron can be released from nitrosylheme at high temperature making it difficult to evaluate the different forms of iron. Thus, nitrosylheme was not studied in the second cooking stage. Concerning nitrite and nitrate, their levels were not affected by the second cooking (results not shown).

3.1. Biochemical composition and molecular interactions in the cured and cooked meat model

3.1.1. Impact of sodium nitrite and ascorbate added on residual nitrite and nitrate

Residual nitrate and nitrite are presented in Table 1. Overall, the increasing doses of added sodium nitrite led to an increase of residual nitrate and nitrite. The basal level of residual nitrate was higher than the values generally cited in the literature for fresh meat. For instance, using the ion chromatography method, Iammarino and di Taranto (2012) found a maximum level of 36.5 ppm of nitrate for red meat, while our results showed a value of 45.7 ppm in the case of 0 ppm+A. The dose-response relationship of added sodium nitrite was similar between 0, 40, and 80 ppm. Only a significant increase of residual nitrite was observed for the 120 ppm+A condition (p=0.001).

Overall, the levels of residual nitrate observed in our study were higher than those frequently reported in the literature. This could be explained by the determination method, in particular the extraction conditions we used. Indeed, (Hamano, Mitsuhashi, Tanaka, Matsuki, Oji, & Okamoto, 2014) demonstrated that deproteinization by ultrafiltration, as performed in our

study, led to an increase of 2.3 times more extracted nitrate than classical chemical deproteinization. Indeed, in chemical deproteinization nitrate can co-precipitate with proteins leading to an underestimation of its concentration. Furthermore, the processing used to prepare the cured and cooked model was chosen to optimize the study of these chemical reactions, and is considered as very oxidative. Indeed, freezing/thawing as well as mixing and cooking, increase oxidation in addition to the using of red meat (shoulder). These different steps could lead to the release of ammonia from oxidized proteins and, in very oxidative medium, ammonia can be oxidized into hydroxylamine by free oxygenated radicals and then into nitrate (Fotiou et al., 2009). Moreover, according to Honikel (2008), products formulated only with nitrite show residual nitrate concentrations (around 1/3 of the added nitrite) in the finished product due to the oxidation of nitrite to nitrate.

Contrary to nitrate, no residual nitrite was observed in the 0 ppm+A model. This result is in good agreement with that of Immarino and di Taranto (2012) and Higuero et al. (2020), who observed nitrate but no nitrite in pork. Nitrite residual content increased significantly (p=0.001) (Table 1) with the sodium nitrite added. This has also been reported by Higuero et al. (2020), who described that the amount of residual nitrite and nitrate increased by increasing the dose of nitrite/nitrate added.

In the cured model with 120 ppm of sodium nitrite, a significant increase of residual nitrite and nitrate was observed when adding ascorbate (p=0.001) (Table 1). In the absence of ascorbate, more nitroso compounds could be formed perhaps explaining the difference observed here. This hypothesis will be confirmed by the results in section 3.1.5., which show considerably more S-nitrosation in the absence of ascorbate.

3.1.2. Impact of sodium nitrite added to ascorbate chemistry

As mentioned in section 2.2.1., sodium ascorbate was added at the level of 300 mg per kg of cured meat, and a control without ascorbate was performed with 120 ppm of sodium nitrite. In our model, only oxidized ascorbate (dehydroascorbate) was observed (Table 1). This oxidation of ascorbate might be explained by its implication in iron and nitrous oxide reduction during the curing process. When no nitrite was added, *i.e.* 0 ppm+A, we observed only 98 ppm of residual dehydroascorbate. This low level could be due to the strong oxidation occurring during cooking and freezing, leading to the irreversible oxidation of dehydroascorbate into deketogulonate (Vieira, Teixeira, & Silva, 2000). A significant (p=0.001) effect of sodium nitrite addition was observed on dehydroascorbate, whose levels varied from 312 to 357 ppm. These values were higher than the dose of 300 ppm added, probably due to the natural ascorbate content in fresh meat and due to the natural decomposition of ascorbate during cooking (Jutkus, Li, Taylor, & Mauer, 2015). For instance, around 24 ppm of ascorbate was measured in fresh pork meat by Lahucky, Bahelka, Novotna and Vasickova (2005). In our study, a value of 42 ppm was observed in the samples without ascorbate added. This higher value, when compared to Lahucky et al. (2005) could be explained by a drip loss during the process, leading to a concentration of ascorbate. This result highlights the protective effect of nitrite against the oxidative degradation of dehydroascorbate into non-vitamin compounds during the curing process.

3.1.3. Impact of sodium nitrite and ascorbate added in non-heme iron chemistry

Total non-heme iron was determined in the cured meat model and each form, *i.e.* reduced (ferrous) and oxidized (ferric) were also quantified (Table 1). The content of non-heme iron was significantly (p=0.0000001) higher when no sodium nitrite was added to the model, *i.e.* 0 ppm+A condition, compared to the conditions with nitrite, regardless of the concentration.

This result can be explained by the release of heme iron during the curing and cooking process in the absence of added sodium nitrite, as will be described in section 3.1.4. PCA, (Supplementary Material), showed that the variables free iron and heme iron were opposite on the first axis and the correlation matrix, showing a significant (-0.81) negative correlation between them and confirming this plausible mechanism. This mechanism implicates oxygenated free radical chemistry, otherwise known as Fenton chemistry. Indeed, it has been described that hydrogen peroxide can release iron from heme through the oxidative cleavage of the porphyrin ring (Amaral et al., 2018). Hydrogen peroxide is naturally present in meat. It can be formed by the oxidation of ferrous iron in the presence of oxygen ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\circ -}$) followed by the dismutation of superoxide ion ($2O_2^{\circ -} + 2H^+ \rightarrow H_2O_2 + O_2$) (for a review on iron catalyst chemistry; Bechaux, de La Pomélie, Théron, Santé-Lhoutellier, & Gatellier, 2018). In presence of nitrite, nitric oxide can react with superoxide radicals to form a stable peroxynitrite ($NO^{\circ} + O_2^{\circ -} \rightarrow ONOO^-$) (Bechaux et al., 2018), thereby preventing the formation of hydrogen peroxide and so increasing heme iron stability. The effect of nitrite was mainly observed between 0 ppm+A and 40 ppm+A and a further increase of sodium nitrite had only a slight effect on the non-heme iron content. The addition of sodium nitrite in the model induced a significant increase of approximatively 10% in ferrous iron between 0 ppm+A and 40 ppm+A, which may be due to the ability of nitric oxide to reduce ferric iron ($Fe^{3+} + NO^{\circ} \rightarrow Fe^{2+} + NO^+$) (Honikel, 2008). The addition of ascorbate at the highest dose of nitrite did not impact the total non-heme iron content (Table 1). However, in the absence of ascorbate, ferric iron is predominant (92%). These results are in agreement with those of Realini et al. (2013) who reported that in red muscles of pigs, non-heme iron is mainly present in ferric form (93%). Indeed, the addition of ascorbate induces the reduction of ferric iron into ferrous iron according to the reaction $Fe^{3+} + Asc^- \rightarrow Fe^{2+} + DHA$.

3.1.4. Impact of sodium nitrite and ascorbate added to heme iron

Total heme iron and nitrosylated-heme iron were quantified in our model and the percentage of nitrosylation was calculated (Fig. 1). In the absence of sodium nitrite, the content of total heme iron was significantly lower (p=0.000001) than in conditions where it was added, regardless of its concentration. As described in section 3.1.3, these results can be explained by the antioxidant capacity of nitrite that prevents heme iron release during the curing and cooking process, supported by a positive correlation between heme iron and nitrite (0.65). We can also hypothesize that this result was due to the stronger stability of nitrosylheme. Indeed, the nitrosylation of heme prevents the release of iron from the porphyrin ring during oxidative stress (Juckett et al., 1998). It is noteworthy that the sum of non-heme and heme iron content led to approximately the same values, whatever the treatment of meat, showing a notable conservation of the iron level in the model. Adding sodium nitrite significantly increased (p=0.000001) nitrosylation without a dose effect. These results are in accordance with Higuero et al. (2020) who described a significant increase in the rate of nitrosomyoglobin in dry-cured loins of between 0 and 37.5 ppm of added nitrite/nitrate, not significantly affect by higher nitrite doses.

The addition of ascorbate did not affect nitrosylation (Fig. 1). Since the process was mainly conducted under vacuum conditions, the formation of deoxymyoglobin ($Mb(Fe^{2+})$) might have been favored, independently of the presence of ascorbate. Deoxymyoglobin is the form in which myoglobin preferentially fixes nitric oxide to form nitrosomyoglobin ($Mb(Fe^{2+}) - NO$) according to the following reaction $Mb(Fe^{2+}) + NO^{\circ} \rightarrow Mb(Fe^{2+}) - NO$ (Richards, 2013). Consequently, the experimental conditions, with a process mainly performed under vacuum, probably made the reducing effect of ascorbate unobservable.

400

401 3.1.5. Impact of sodium nitrite and ascorbate added on nitrosation reactions

402

403 The products of nitrosation, *i.e.* nitrosothiols and nitrosamines, were measured in the cured
404 and cooked meat (Fig. 2). The non-volatile nitrosamines were not detected except at a low
405 value of 0.2 ppm for 40 ppm of added sodium nitrite. In nitrite-cured pork meat, Van Hecke,
406 Vossen, Vanden Bussche, Raes, Vanhaecke, & De Smet, 2014 did not show an NOC-induced
407 DNA adduct, demonstrating the very low level of nitrosation during the process. Herrmann et
408 al. (2015a) found a low level of nitrosamines in smoked ham, between 0.03 ppm of non-
409 volatile N-nitroso-methylaniline (NMA) and 2 ppm of non-volatile nitrosamine N-nitroso-
410 thiazolidine-4-carboxylic acid (NTCA). These authors also found up to 0.5 ppm of non-
411 volatile nitrosamine N-nitroso-thiazolidine-4-carboxylic acid (NTCA) in bacon and 4 ppm in
412 salami. Moreover, according to Massey, Key, Jones and Logan (1991) the Apparent Total N-
413 Nitroso Compounds levels in bacon are in the range of 0.4 to 6.8 ppm (N-NO). Nevertheless,
414 all these products underwent manufacturing processes (smoking, drying and salting) that
415 differed from our model, probably favoring the formation of nitrosamines, which could
416 explain these higher values.
417 On the contrary, nitrosothiol content increased with sodium nitrite addition, with a significant
418 effect observed at 80 ppm. Joosen et al. (2009) reported up to 22 ppm of nitrosothiols in
419 bacon, a value higher than those observed in our samples which ranged from 0.5 to 4 ppm.
420 The smoking step applied to the bacon could have promoted the formation of NOCs.
421 At 120 ppm of sodium nitrite added without ascorbate, a six-fold increase (p=0.0000001) of
422 nitrosothiols was observed when compared to the corresponding samples with ascorbate,
423 while no nitrosamines were observed. This result clearly shows a protective effect of
424 ascorbate against the nitrosation process. In an acidic condition and in the presence of

ascorbate, nitrosylation was found to predominate over nitrosation (Honikel, 2008; Skibsted, 2011). This was due to the formation of nitrous acid ($NO_2^- + H^+ \rightarrow HNO_2$) followed by its reduction into nitric oxide ($HNO_2 + H^+ + e^- \rightarrow NO^\circ + H_2O$) while, in the absence of ascorbate, nitrosation was favored by the formation of nitrosonium from nitrous acid ($HNO_2 + H^+ \rightarrow NO^+ + H_2O$). Consequently, we can hypothesise that in our model, nitrosothiols were predominantly formed by the reaction of nitrosonium with the free thiols. Moreover, the correlation matrix (Supplementary Material), showed a negative correlation (-0.52) between nitrosothiols and ascorbate in its oxidation form (dehydroascorbate), which was the only form observed in the model. In addition, a possible increase of protective effect of ascorbate against nitrosation during the process could be assumed. Indeed, both ascorbate and dehydroascorbate can react with nitrous acid to form (di)nitroso-ascorbic/dehydroascorbic acid intermediates (Skibsted, 2011), thereby preventing the reaction of a nitrite derivate with free thiols.

3.1.6. Impact of sodium nitrite and ascorbate added in oxidation mechanisms

The lipid oxidation determination ranged from 6 µg of MDA per g of lipids for the 120 ppm+A to 18 µg of MDA for the no nitrite condition (Fig. 3). These values correspond to approximately 0.5 and 1.5 mg MDA/kg of meat. These values are below the strong rancid smell threshold generally reported in the literature, from 1.5 mg MDA/kg of meat (Tarladgis, Watts, Younathan, & Dugan, 1960). So, all these products were available for consumption. In the 0 ppm+A model, lipid oxidation was significantly (p=0.0000001) higher than in other conditions. This difference can be explained by nitrite, which acts as a powerful antioxidant in such products (Berardo et al., 2016). Our results are in line with those reported for dry-cured loins (Higuero et al., 2020) and cooked cured meat products (Karwowska, Kononiuk &

Wojciak, 2019; Santarelli, et al., 2010). Also, as described in section 3.1.3., the absence of nitrite leads to the increase of free iron. Free iron is known to promote lipid oxidation by the Fenton reaction, leading to the formation of oxygenated free radicals, and by reacting with lipoperoxides to form alcoxyle and peroxyle free radicals ($Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\circ} + OH^{-}$) and ($Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO^{\circ} + H^{+}$) (Bechaux et al., 2018) . Moreover, nitric oxide reacts with superoxide radicals, as previously described in section 3.1.3., and lipid radicals to form stable LONO and unstable LOONO that decomposes into stable $LONO_2$ ($NO^{\circ} + L(O)O^{\circ} \rightarrow$ L(O)ONO) (Bechaux et al., 2018), thereby ending the oxidation process. The addition of nitrite decreases lipid oxidation but no significant dose-response relationship was observed. These mechanisms were confirmed by PCA and the correlation matrix, (Supplementary Material), showing a positive correlation (0.78) of TBARS and non-heme iron. On the contrary, a significant negative correlation (-0.73) was observed between TBARS and residual nitrite. Van Hecke et al. (2014) described a considerable decrease of lipid peroxidation in cured and cooked meat, with 120 ppm of nitrite added, when compared to uncured meat. Moreover, a gradual decrease in TBARS values with the increase in amount of sodium nitrite added (from 0 to 150 ppm) was previously described by Karwowska et al. (2019) in cooked meat products. Free thiol content was also estimated. Contrary to lipid oxidation, no nitrite effect was observed for thiols oxidation (results not shown). This was probably due to considerable thiol oxidation in the previous steps of the process (freezing, thawing and mincing).

The addition of ascorbate did not affect lipid oxidation (Fig. 3). It is more likely that the high antioxidant power of nitrite, described above, masked the antioxidant effect of ascorbate. In the conditions of the process applied, no greater effect of nitrite and ascorbate was observed on lipid oxidation. These results are in agreement with those of Berardo et al. (2016) who did not report a higher effect of nitrite and ascorbate when compared to the separate addition of

475 nitrite or ascorbate on lipid oxidation in dry fermented sausages. Moreover, ascorbate can act
476 as a pro-oxidant (Amaral et al., 2018), especially in the presence of a high amount of iron, by
477 regenerating the ferrous form, which acts in the reactions previously described. In addition,
478 ascorbate did not affect thiol oxidation (results not shown).
479
480 3.2. Effect of sodium nitrite doses and ascorbate on nitrosation during the second cooking
481 stage of the cured and cooked meat model
482
483 Cured meat products are sometimes recooked at high temperature before consumption, such
484 as ham in pizza toppings. Sausages and bacon are also often grilled or fried at high
485 temperature. Thus, it is of great interest to evaluate the extent of nitrosation that occurs
486 during these cooking processes. A significant increase of non-volatile nitrosamines was
487 observed after the second cooking stage (p=0.0000001) and sodium nitrite addition
488 (p=0.00007) (Fig. 4A). Nitrosamines increased significantly (p=0.00007) between 0 and 40
489 ppm of added sodium nitrite and the further increase was not significant. An high increase of
490 nitrosamine content in dried and fried sausages with increasing doses of nitrite from 0 to 350
491 ppm was already described by Herrmann et al. (2015b). Such high levels of nitrosamines
492 could negatively impact the health quality of cured meat. Thus, to prevent the formation of
493 these unsuitable products it is necessary to better understand the reactions involved in their
494 formation during the second cooking process. This is why different mechanisms are
495 described in this paper to better understand the effect of thermal processes on nitrosation.
496 The nitrosothiol content increased significantly (p=0.000001) when increasing the sodium
497 nitrite content (Fig. 4B). But, contrary to nitrosamines, no second cooking effect was
498 observed on nitrosothiols. The absence of a second cooking effect could be due to the low
499 stability of nitrosothiols at high temperature. Indeed, nitrosothiols begin to decompose from

130°C upwards to release NO (Melvin, Jones, Lutzke, Allison & Reynolds, 2019). In contrast, most non-volatile nitrosamines are stable at high temperature; their boiling point generally exceeds 200°C. For example, the most common non-volatile nitrosamines N-nitrososarcosine (BP 221°C) and N-nitrosoproline (BP 263°C), are particularly thermostable. Nitrosothiols are not described in the literature as dangerous by themselves, but transnitrosation from nitrosothiols to secondary amines (Wainright, 1986) during digestion is possible with, in this case, the risk of formation of mutagenic compounds (Kuhnle & Bingham, 2007).

One of the ways described in the literature to reduce nitrosation is the addition of ascorbate to products. This is why we decided to evaluate during the second cooking of cured meat the impact of dehydroascorbate, which was the only form of ascorbate after the initial cooking (Table 1). Ascorbate is thermosensitive, and its activity rapidly decreases after 95°C. Nevertheless, dehydroascorbate, is more resistant to thermal treatment (Vieira et al., 2000) and considerable residues were observed after the second cooking. These residues were measured at 58%, 93% and 22% for the 180°C for 7 min, 220°C for 6 min and 260°C for 4 min treatments, respectively.

During the second cooking stage, dehydroascorbate limited the formation of nitrosamines (Fig. 5A) especially for the condition 180°C for 7 min (p=0.007). These results were in agreement with those of Mottram, Patterson, Rhodes & Gough (1975), who showed a reduction in the production of N-nitrosodimethylamine (NDMA) in the presence of ascorbate. In addition, Herrmann et al. (2015b) showed the inhibitory effect of antioxidants, such as erythorbate and ascorbate, on the formation of certain nitrosamines during heating treatments. The mechanisms involved in this protective effect have not yet been thoroughly described, but ascorbate, as well as dehydroascorbate, could react with nitrous acid to form (di)nitroso-ascorbate or (di)nitroso-dehydroascorbate intermediates (Skibsted, 2011), possibly preventing

N-nitrosation. This study did not show a significant effect between the three different time/temperature pairs on the nitrosamine content. Therefore, our study does not distinguish the best time/temperature combination of second cooking in terms of consumer health.

Nitrosothiol content was significantly lower (p=0.000001) in the absence of dehydroascorbate for each second cooking condition (Fig. 5B). This was significantly (p=0.0057) more pronounced at 180°C for 7 min. Hence, this result showed a dehydroascorbate effect contrary to that observed on nitrosamines in the second cooking stage, and also a contrary effect to that observed on nitrosothiols in the cured and cooked meat model (section 3.1.5.). This could be explained by a mechanism differing from that described previously. During second cooking, dehydroascorbate could protect nitrosothiols from reaction with transition metals that cleave the S-NO bond. These transition metals are released at high temperature: iron is released in large amounts from ferritin and myoglobin while copper is released to a lesser extent from various enzymes such as (Cu/Zn)-Superoxide dismutase and cytochrome C oxidase. Ascorbate and dehydroascorbate are iron and copper chelators (Fisher & Naughton, 2004) and so they could stabilize nitrosothiols (Melvin et al., 2019).

As previously observed with nitrosamines, the second cooking stage did not show a difference between the three time/temperature pairs on nitrosothiol content.

We can also notice that the increase of the molar nitrosamine concentration in the absence of ascorbate was not significantly different from the decrease of the molar nitrosothiol concentration in the same condition (results not shown), so that global nitrosation (N + S nitrosation) was not affected by ascorbate. This observation could suggest a possible transfer of NO groups from nitrosothiols to nitrosamines during the second cooking stage in the absence of ascorbate. Such transnitrosation was already observed in cured meat by Wainright (1986).

4. Conclusion

This study gives new insights on the formation of nitroso compounds in cured cooked meat products. The absence of nitrite leads to a decrease of nitrosylheme but promotes the release of heme iron from myoglobin. The iron released is then implicated in the formation of free radicals, involving the formation of oxidation products, some of which are mutagenic. The addition of nitrite promotes the formation of nitroso compounds without a dose-response effect. However, we also observed the role of nitrite as an antioxidant, preventing lipid oxidation and to the ascorbate degradation during the curing process. A second cooking stage promotes the formation of non-volatile nitrosamines. Ascorbate limits the formation of these compounds at moderate high temperature (180°C) but at the same time protects the nitrosothiols from degradation.

This study contributes to elucidating the chemical reactivity of NOCs in a cured meat model. The results obtained here will help to better understand the behavior of cured meat products during digestion, and more specifically NOCs. Indeed, the physicochemical conditions of the digestive tract, like oxygen pressure, low gastric pH, and reducing conditions, favor the formation of nitrite derivatives such as nitrosonium ions and nitric oxide that promote the formation of nitroso compounds. We will also study ways of limiting the formation of these compounds in the digestive tract.

Conflict of interest

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705

Figure caption

Table 1. Effect of added doses of sodium nitrite and ascorbate in the cured and cooked meat model on the concentration of residual nitrite, nitrate, ascorbate and free iron. 0ppm+A; 40ppm+A; 80ppm+A; 120ppm+A correspond respectively to 0, 40, 80 and 120 ppm of sodium nitrite added in the presence of ascorbate. Samples 120ppm-A correspond to 120 ppm of sodium nitrite added without ascorbate. The effect of nitrite and ascorbate treatment in the cooked model were assessed independently by a one-way analysis of variance with Tukey post hoc test.

Figure 1. Effect of added sodium nitrite doses and ascorbate on the concentration of nitrosylated heme iron and total heme iron, expressed in mM, in the cured and cooked meat model. 0ppm+A; 40ppm+A; 80ppm+A; 120ppm+A correspond respectively to 0, 40, 80 and 120 ppm of sodium nitrite added in the presence of ascorbate. Samples 120ppm-A correspond to 120 ppm of sodium nitrite added without ascorbate. The effect of nitrite and ascorbate treatment in the cooked model were assessed independently by a one-way analysis of variance with Tukey post hoc test.

Figure 2. Effect of added sodium nitrite doses and ascorbate on the concentration of nitrosation products, expressed in ppm (mg/ kg of wet matter): nitrosothiols and nitrosamines in the cured and cooked meat model. 0ppm+A; 40ppm+A; 80ppm+A; 120ppm+A correspond respectively to 0, 40, 80 and 120 ppm of sodium nitrite added in the presence of ascorbate. Samples 120ppm-A correspond to 120 ppm of sodium nitrite added without ascorbate. The effect of nitrite and ascorbate treatment in the cooked model were assessed independently by a one-way analysis of variance with Tukey post hoc test.

Figure 3. Effect of added sodium nitrite doses and ascorbate on the concentration of TBA-RS in the cured and cooked meat model, expressed in µg MDA/ g lipids. 0ppm+A; 40ppm+A; 80ppm+A; 120ppm+A correspond respectively to 0, 40, 80 and 120 ppm of sodium nitrite added in the presence of ascorbate. Samples 120ppm-A correspond to 120 ppm of sodium nitrite added without ascorbate. The effect of nitrite and ascorbate treatment in the cooked model were assessed independently by a one-way analysis of variance with Tukey post hoc test.

Figure 4. Effect of added sodium nitrite doses in the cured and cooked meat model before and after second cooking (220°C/ 6 min) on nitrosamine (A) and nitrosothiol (B) concentrations expressed in ppm (mg/ kg dry matter). 0ppm+A; 40ppm+A; 80ppm+A; 120ppm+A correspond respectively to 0, 40, 80 and 120 ppm of sodium nitrite added in the presence of ascorbate. The effect of nitrite in the cooked model were assessed independently by a one-way analysis of variance with Tukey post hoc test. The effect of second cooking conditions and sodium nitrite doses were tested together by a two-way analysis of variance.

Figure 5. Effect of different second cooking conditions (180°C for 7 min, 220°C for 6 min, 260°C for 4 min), with ascorbate (in white) and without ascorbate addition (in grey) (with 120 ppm sodium nitrite) on nitrosamine (A) and nitrosothiol (B) concentrations expressed in ppm (mg/ kg dry matter). The effect of nitrite and ascorbate treatment in the cooked model were assessed independently by a one-way analysis of variance with Tukey post hoc test. The effect of second cooking conditions and ascorbate addition were tested together by a two-way analysis of variance.

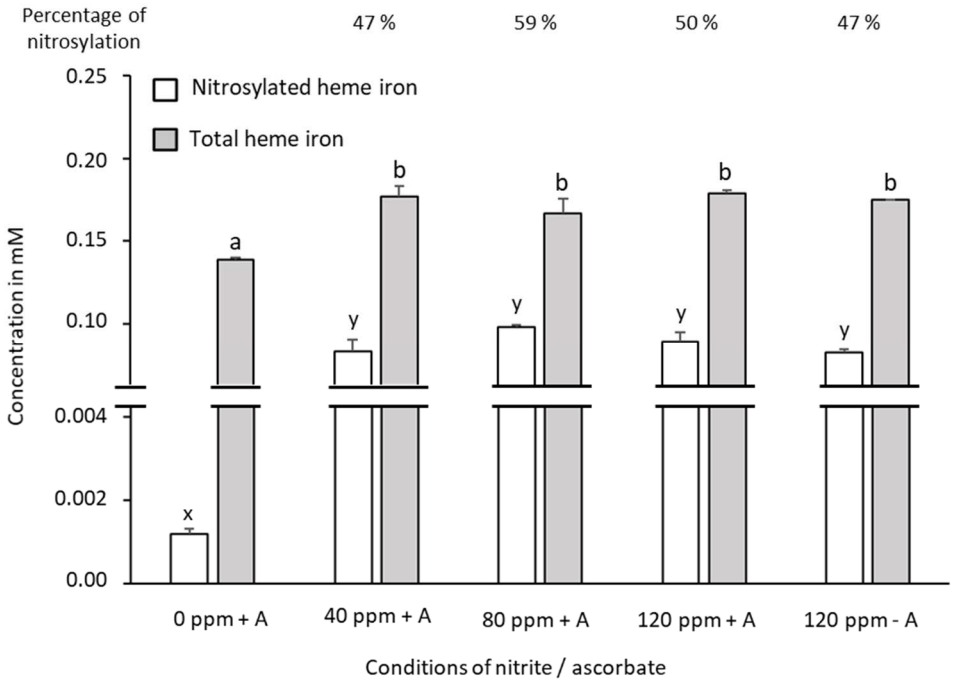

Figure 1. Effect of added sodium nitrite doses and ascorbate on the concentration of nitrosylated heme iron and total heme iron, expressed in mM, in the cured and cooked meat model. Values are mean ± SEM of 6 independent determinations. Values without common superscripts, a, b for total heme iron and x, y for nitrosylated heme iron, differ significantly ($p < 0.01$).

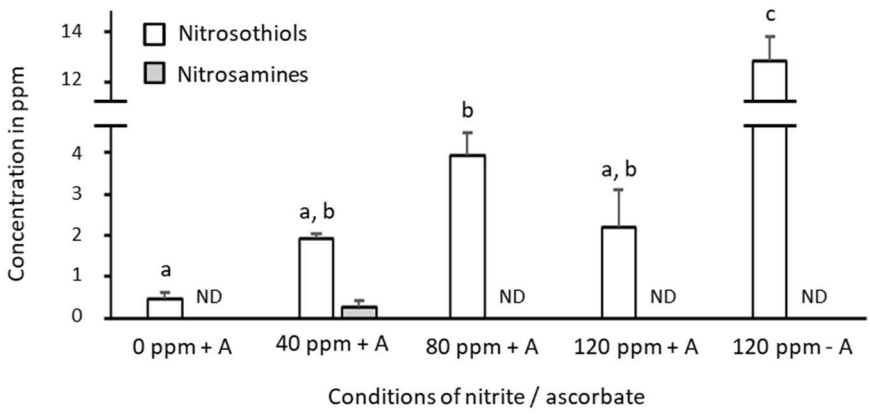

Figure 2. Effect of added sodium nitrite doses and ascorbate on the concentration of nitrosation products, expressed in ppm (mg/ kg of wet matter): nitrosothiols and nitrosamines in the cured and cooked meat model. Values are mean ± SEM of 6 independent determinations. Values without common superscripts, a, b, c for total nitrosothiols, differ significantly ($p < 0.01$). ND for not detected.

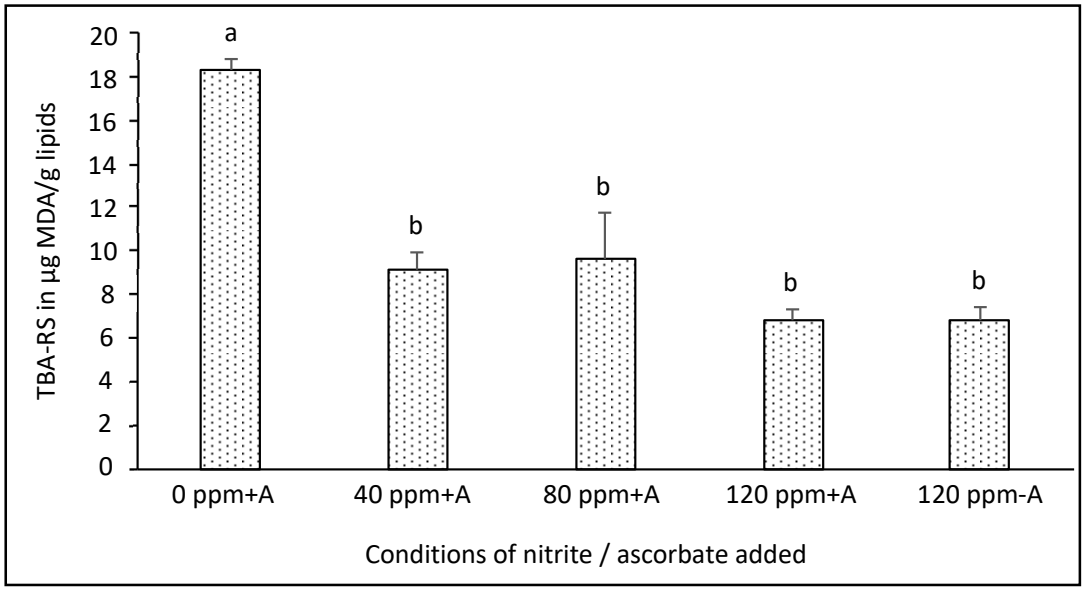

Figure 3. Effect of added sodium nitrite doses and ascorbate on the concentration of TBA-RS in the cured and cooked meat model, expressed in µg MDA/ g lipids.

Values are mean ± SEM of 6 independent determinations. Values without common superscripts differ significantly ($p < 0.01$).

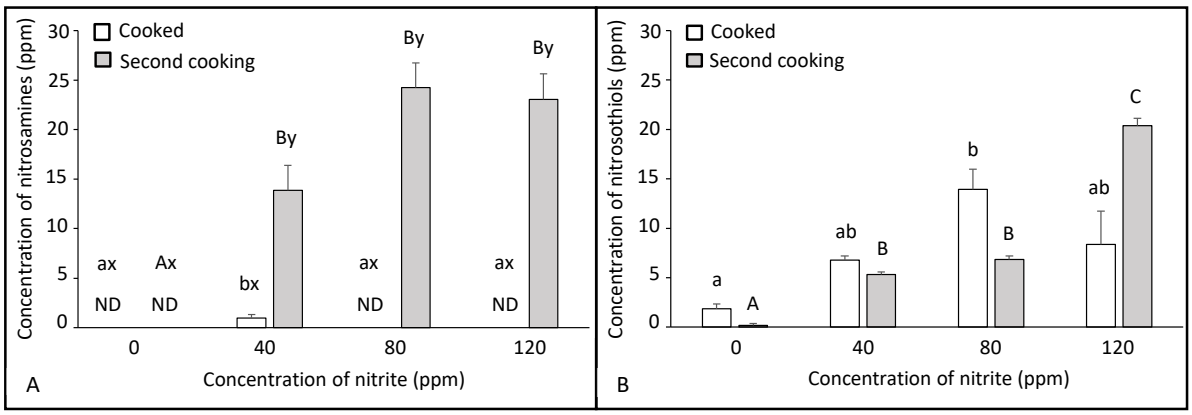
Concentration of nitrosamines (ppm)
Cooked
Second cooking
30
25
20
15
10
5
0
ax
ND
Ax
ND
bx
By
ax
ND
By
ax
ND
By
0
40
80
120
Concentration of nitrite (ppm)
A
Concentration of nitrosothiols (ppm)
Cooked
Second cooking
a
A
ab
B
b
B
ab
C
0
40
80
120
Concentration of nitrite (ppm)
B

Figure 4.

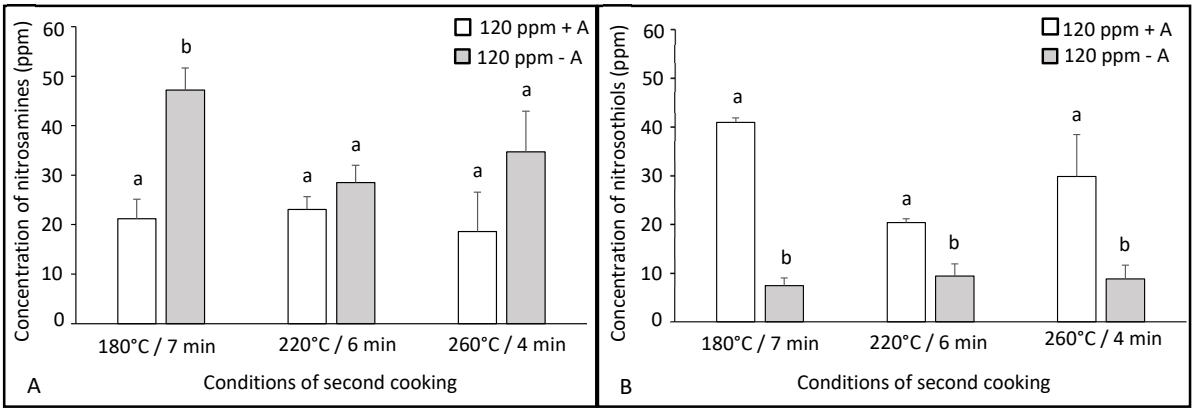
Concentration of nitrosamines (ppm)
60
50
40
30
20
10
0
120 ppm + A
120 ppm - A
a
b
a
a
a
a
180°C / 7 min
220°C / 6 min
260°C / 4 min
A
Conditions of second cooking
Concentration of nitrosothiols (ppm)
60
50
40
30
20
10
0
120 ppm + A
120 ppm - A
a
b
a
b
a
b
180°C / 7 min
220°C / 6 min
260°C / 4 min
B
Conditions of second cooking

Figure 5.

Table 1. Effect of added doses of sodium nitrite and ascorbate in the cured and cooked meat model on the concentration of residual nitrite, nitrate, ascorbate and free iron.

	0 ppm+A	40 ppm+A	80 ppm+A	120 ppm+A	120 ppm-A
Residual NO_2 (ppm)	$0.00^{a} \pm 0.00$	$7.74^{b} \pm 0.16$	$18.33^{c} \pm 0.64$	$34.28^{e} \pm 1.75$	$29.91^{d} \pm 1.43$
Residual NO_3 (ppm)	$45.68^{a} \pm 14.04$	$61.60^{a} \pm 3.87$	$47.52^{a} \pm 7.55$	$120.86^{c} \pm 6.78$	$84.16^{b} \pm 10.74$
Oxidized ascorbate (ppm)	$98.30^{a} \pm 20.98$	$312.39^{b} \pm 19.90$	$357.09^{b} \pm 19.82$	$340.19^{b} \pm 12.57$	$41.93^{a} \pm 12.95$
Free iron total (µM)	$82.07^{c} \pm 1.73$	$28.45^{a} \pm 1.61$	$26.66^{a} \pm 0.87$	$34.95^{b} \pm 1.20$	$35.49^{b} \pm 0.78$
Ferrous iron Fe^{2+} (%)	$60.73^{b} \pm 0.60$	$71.25^{c} \pm 5.46$	$66.87^{c} \pm 1.26$	$72.85^{d} \pm 0.34$	$7.96^{a} \pm 4.12$
Ferric iron Fe^{3+} (%)	$39.27^{c} \pm 0.62$	$28.75^{b} \pm 5.46$	$33.13^{b} \pm 1.26$	$27.15^{a} \pm 0.34$	$92.04^{d} \pm 4.12$