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Effect of sodium ascorbate and sodium nitrite on protein and lipid oxidation in dry fermented sausages

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ABSTRACT

The effects of sodium nitrite and ascorbate on lipid and protein oxidation were studied during the ripening process of dry fermented sausages. Samples were taken at day 0, 2, 8, 14, 21 and 28 of ripening to assess lipid (malondialdehyde) and protein (carbonyls and sulfhydryl groups) oxidation. Sodium ascorbate and nitrite were separately able to reduce the formation of malondialdehyde. Their combined addition resulted in higher amounts of carbonyl compounds compared to their separate addition or the treatment without any of both compounds. Moreover, sodium nitrite limited the formation of γ-glutamic semialdehyde whereas sodium ascorbate showed a pro-oxidant effect. A loss of thiol groups was observed during ripening, which was not affected by the use of sodium ascorbate nor sodium nitrite. In conclusion, sodium nitrite and ascorbate affected protein and lipid oxidation in different manners. The possible pro-oxidant effect of their combined addition on carbonyl formation might influence the technological and sensory properties of these products.

1. Introduction

The stable character of dry fermented sausages is largely due to a combination of salting, bacterial acidification, drying and sometimes smoking. The salting process includes the addition of sodium chloride, nitrate and/or nitrite salts, and ascorbate salts. Nitrite and ascorbate salts are basic ingredients in fermented meat products. Nitrite can also be bacterially derived from nitrate (Sánchez Mainar & Leroy, 2015). In combination, these ingredients develop the desired red colour (Alley, Cours, & Demeyer, 1992) and the cured flavour in fermented products (Toldra et al., 2009). Moreover, nitrite exerts antimicrobial activity (Cassens, 1990).

The chemistry of nitrite and ascorbate in processed meat products is complex and not fully understood yet. Although nitrite is a natural electron acceptor and hence a potential oxidizing agent (Villaverde, Parra, & Estévez, 2014a), the ability of this compound to prevent lipid oxidation in meat products is well established (Balev, Vulkova, Dragoev, Zlatanov, & Bahtchevanska, 2005; Zanardi, Ghidini, Battaglia, & ChizzoliniR, 2004). Ascorbate is also involved in redox reactions; this compound is an electron donor and its oxidized form (dehydroascorbic acid) is relatively unreactive and therefore terminates the propagation of free radical reaction (Bendich, Machlin, Scandurra, Burton, & Wayner, 1986). Nevertheless, ascorbate can act as pro-oxidant in the presence of metal ions. Indeed, its ability to reduce metal ions promotes the generation of reactive oxygen species through the Fenton reaction (Villaverde et al., 2014a). Hence, it has been shown that the use of ascorbate salts in processed meats inhibits lipid oxidation (Balev et al., 2005), but pro-oxidant effects have been reported as well (Haak, Raes, & De Smet, 2009).

Proteins together with lipids are important constituents of meat products and undergo oxidation too. However, the effects of nitrite and ascorbate on protein oxidation have been much less investigated. Protein oxidation is potentially important for meat fermentation since it implies modifications at the protein level which can alter the structure and functionality of proteins, compromising their technological and sensory properties (Lund, Heinonen, Baron, & Estévez, 2011). Firstly, the reaction between lipid oxidation products and protein amines generates Schiff bases which may affect colour and flavour (Chelh, Gatellier, & Sante-Lhoutellier, 2007). Secondly, oxidation of proteolytic enzymes may compromise their activity and indirectly influence the flavour (Berardo, Claeys, Vossen, Leroy, & De Smet, 2015). Thirdly, the formation of crosslinks between proteins that are affected by protein oxidation may affect the texture of fermented sausages, in particular with respect to gelation (Zhou, Zhao, Zhao, Sun, & Cui, 2014).

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Considering the above-mentioned knowledge gap, the aim of the present study was to investigate the effects of sodium nitrite and sodium ascorbate on the oxidation of both lipids and proteins during ripening in dry fermented sausages.

2. Materials and methods

2.1. Dry fermented sausage preparation

Dry fermented sausages were prepared mixing lean pork (70.5%), pork backfat (27.0%), sodium chloride (2.5%) and a starter culture containing a mixture of *Lactobacillus sakei* CTC 494, *Staphylococcus carnosus* 833 and *Staphylococcus xylosus* 2S7-2 (Janssens et al., 2014; Ravyts et al., 2010). Sodium ascorbate (SA) and sodium nitrite (SN) were added according to a 2 × 2 factorial design with the following four treatments: 1) a control treatment without sodium ascorbate and sodium nitrite (Control); 2) sodium ascorbate added at 500 mg/kg without sodium nitrite (SA); 3) sodium nitrite added at 150 mg/kg without sodium ascorbate (SN); 4) sodium ascorbate added at 500 mg/kg and sodium nitrite at 150 mg/kg (SA + SN). The batter was stuffed into collagen casings of 50 mm diameter (Naturin, Weinheim, Germany) and ripened for 28 days in a climate chamber (Kerres Anlagensysteme GmbH, Backnang, Germany). During the first two days, fermentation was performed at a temperature of 24 °C and a relative humidity of 94%. For the drying process, the temperature was dropped to 12 °C and relative humidity was set to 82% after the first two weeks. Samples were taken after 0 (day of production), 2 (end of fermentation), 8, 14, 21, and 28 (end of ripening) days. At each sampling day, one sausage per treatment was taken for analysis (except for pH and weight loss which were measured in three sausages per treatment throughout the ripening as described below). The manufacturing process was repeated once on a separate day, resulting in two independent replicate batches and samples.

2.2. pH and weigh loss

In each manufacturing process, three randomly selected sausages per treatment were weighed and the pH was recorded after their preparation and during ripening. The pH was measured directly in the sausages [ISO 2917 (1999)] and the pH meter was calibrated in buffers of pH 4.0 and 7.0. Weight loss was expressed as a percentage of the initial weight and the mean of the three records was calculated.

2.3. Residual ascorbic acid (AA)

Residual AA was determined through high-performance liquid chromatography (HPLC) based on the reaction of dehydroascorbic acid with orthophenylenediamine (OPD) as described by Doolaege et al. (2012). Briefly, AA and dehydroascorbic acid (DHAA) were extracted using methanol/water (5/95; v/v) containing 0.1 M citric acid and 0.2 mM EDTA. DHAA is able to react with OPD but AA needs to be converted into DHAA first, using active carbon. By measuring total DHAA (i.e. present DHAA and DHAA formed from converted AA), and DHAA present in the samples, the AA concentration was calculated. Samples were analysed by reversed phase HPLC [150 × 4.6 mm Nucleosil 100 C18 column (3 μm) (Grace Davison Discovery Sciences, Lokeren, Belgium)] with fluorimetric detection (Agilent, Waldbronn, Germany) using excitation and emission wavelengths of 350 and 430 nm, respectively. The mobile phase was a mixture of methanol/water (5/95; v/v), containing 5 mM cetrimide and 50 mM·KH_2PO_4 (pH 4.6). The elution was performed at a flow rate of 1.0 mL/min. Quantification was done by comparison of peak areas with those obtained from a standard solution of converted AA. Results were expressed in mg AA/kg sample.

2.4. Residual nitrite

Residual nitrite was determined according to ISO Standard 2918. Briefly, 4 g of sample was homogenized in 50 mL NaOH 0.02 M with 0.2 g of active carbon and incubated for 2 h in a shaking water bath at 80 °C. Then, 5 mL of $ZnSO_4$ was added and the homogenate was cooled to room temperature. The homogenate was then centrifuged at 1670 × *g* for 5 min. The supernatant was diluted to 100 mL with NaOH 0.02 M and filtered through a folded paper filter. Hundred μL of colour reagent A (0.2 g *N*-1-naftylethyleendiamine·2HCl in 150 mL 15% acetic acid) and 100 μL of colour reagent B (0.5 g sulfanilamide in 100 mL 15% acetic acid and 5 mL HCl 12 M) were mixed to 2.5 mL of supernatant or to 2.5 mL NaOH 0.02 M (blank). The absorbance was measured at 538 nm after 15 min. Residual nitrite in mg $NaNO_2$/kg of sample was calculated using a standard curve.

2.5. Lipid oxidation

Lipid oxidation was determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS) as described by Doolaege et al. (2012). In brief, 5 g of meat was homogenized in 40.0 mL $HClO_4$ (0.6 M) and 1.0 mL butylated hydroxytoluene (BHT) solution. The homogenate was filtered and 5.0 mL was transferred in heat resistant glass test tubes together with 1 mL of TBA reagent. The resulting solutions were put in a boiling water bath for 35 min. They were subsequently cooled to room temperature and the absorbance was measured at 532 nm. Lipid oxidation was calculated using a standard curve and expressed as mg malondialdehyde equivalents (MDA eq.)/kg sample.

2.6. Protein carbonyl content

The protein carbonyl content was determined by derivatization with 2,4-dinitrophenyl hydrazine (DNPH) as described by Ganhão, Morcuende, and Estévez (2010). Briefly, 3 g of meat with 30 mL of phosphate buffer (20 mM, pH 6.5 containing 0.6 M NaCl) was homogenized and four aliquots of 0.2 mL were treated with 1 mL ice-cold TCA (10%) to precipitate the proteins. After centrifugation the supernatant was discarded and two aliquots were treated with 0.5 mL of 10 mM DNPH dissolved in 2.0 M HCl and two aliquots were treated with 0.5 mL of 2.0 M HCl (blank). After 1 h of reaction, 0.5 mL of ice cold 20% trichloroacetic acid (TCA) was added. The samples were then centrifuged and supernatant was discarded. Excess DNPH was removed by washing three times with 1 mL of ethanol:ethylacetate (1:1, v/v). The pellets were dissolved in 1 mL of 6.0 M guanidine hydrochloride in 20 mM phosphate buffer (pH 6.5). The carbonyl concentration (nmol/mg protein) was calculated from the absorbance at 280 nm and 370 nm of the samples using the following equation (Levine, Williams, Stadtman, & Shacter, 1994):

$$\frac{C_{hydrazone}}{C_{protein}} = \frac{A_{370}}{\varepsilon_{hydrazone,370} \times (A_{280} - A_{370} \times 0.43)} \times 10^6$$

where $\varepsilon_{hydrazone,370}$ is 22,000 $M^{-1}\cdot cm^{-1}$ and the carbonyl concentrations obtained from the blanks were subtracted from the corresponding treated sample.

2.7. Thiol concentration of myofibrillar proteins

The thiol concentration of myofibrillar proteins was determined after derivatization by Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), following a protocol adopted from Jongberg, Torngren, Gunvig, Skibsted, and Lund (2013). Two grams of frozen meat were homogenized in 30 mL of TRIS buffer (pH 8.0) and centrifuged for 20 min at 1670 × *g*. The supernatant, containing sarcoplasmic proteins and fats, was discarded. The pellet, made up of myofibrillar proteins, was