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Vitamin (B1, B2, B3 and B6) content and oxidative stability of *Gastrocnemius* muscle from dry-cured hams elaborated with different nitrifying salt contents and by two ageing times

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ABSTRACT

The effect of the amount of added nitrate and nitrate plus nitrite to dry-cured hams on the vitamin (B1, B2, B3, B6) content, the antioxidant enzyme superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) activities and the thiobarbituric acid reactive substances (TBARS) was assessed in *Gastrocnemius* muscle at the end of two ripening processes. Five different curing mixtures (Hi–N: 600 KNO_3; Lo–N: 150 KNO_3; Hi–Mix: 600 KNO_3 + 600 $NaNO_2$; Lo–Mix: 150 KNO_3 + 150 $NaNO_2$; Hi–Mix/Asc: 600 KNO_3 + 600 $NaNO_2$ + 500 sodium ascorbate, expressed as mg of salts added on surface per kg of fresh ham) were evaluated in dry-cured hams aged for 11.5 months (standard process, SP) and 22 months (long process, LP). Minor differences in target parameters between the hams due to the process were found. The amount of nitrate when it was added alone or as a mixture of nitrate and nitrite, as well as the ascorbate addition to dry-cured hams did not affect vitamin B1, B2 and B3 contents. The level of vitamin B6 was affected by both the amount and the mixture of salts; the addition of nitrite reduced around 40% the content of vitamin B6, but it was not affected by nitrate or ascorbate. The activity of SOD and CAT decreased with the amount of nitrate and nitrite, while GSHPx and TBARS resulted unaffected.

1. Introduction

Nitrate and nitrite are traditionally used as curing salts in the elaboration of dry-cured ham. Nitrate could be transformed to nitrite by the action of nitrate reductase enzymes present in some microorganisms (e.g. *Staphylococcus* sp.) acting as a nitrite reservoir. Nitrite plays important roles on safety and quality of dry-cured hams inhibiting the growth of spoilage and pathogenic microorganisms (e.g. *Clostridium botulinum*), delays oxidative rancidity and contributes to achieve the typical cured colour and flavour (Arnau, Guerrero, & Gou, 2003; Honikel, 2008; Toldrá, Aristoy, & Flores, 2009). On the other hand, in meat products, a proportion of nitrites and nitrates may be transformed into nitrosamines (Cammack et al., 1999) which are recognised carcinogens (Martínez, Haza, & Morales, 2000a,b). However, reported levels of nitrosamines in dry-cured hams are generally very low (Demeyer et al., 2000).

Nitrates and nitrites are a source of nitric oxide (NO) that can interact with superoxide radical to form the peroxynitrite radical ($ONOO^-$) (Burdon, 1995), a potent cytotoxic oxidant (Pryor & Squadrito, 1995; Szabo, 2003). Superoxide radical and related reactive oxygen (ROS) and nitrogen (RNS) species can be scavenged by the antioxidant enzymatic system and by antioxidant compounds such as sodium ascorbate and tocopherols (Mirvish, 1995; Wagner et al., 1985). Ascorbate is added to meat products to contribute to the meat colour development and to reduce potential nitrosamine formation (Chow & Hong, 2002). In some countries (e.g. USA) it is mandatory to add ascorbate or erythorbate in pumped bacon when nitrifiers are used (Code of Federal Regulations, 2005).

The use of nitrate and nitrite in meat products is regulated in the European Union by the Regulation 1129/2011 of 11 November 2011. The legislation is addressed to lowering the nitrite addition to the minimum necessary to achieve the required preservative effect and ensure microbiological safety. In dry-cured hams, only a part of the added nitrate is absorbed (Arnau, Guerrero, Casademont, & Gou, 1995); for this reason, only the maximum residual levels are regulated, which are 250 mg kg^{-1} (expressed as $NaNO_3$) for nitrate (Commission of Regulation (EU), 1129/2011).

Meat and meat products are known to have an important nutritional value as source of proteins and vitamins of the B group. No published information was found about the effect of curing agents on these nutritional food components in dry-cured hams. Thus, the aim of this work was to study the effect of the addition of different amounts of nitrate and a mixture of nitrate and nitrite to dry-cured ham manufactured with two different ageing times on vitamins B1, B2, B3 and B6. Furthermore, due to the properties of nitrates, nitrites and ascorbate, the oxidative stability was also evaluated by the determination of the main antioxidant enzyme activities superoxide dismutase

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(SOD), catalase (CAT), glutathione peroxidase (GSHPx), and by the thiobarbituric acid reactive substances (TBARS).

2. Material and methods

2.1. Reagents

Reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase (GR), superoxide dismutase (SOD), pyridoxine, riboflavin, nicotinamide, thiamine and taka-diastase from *Aspergillus oryzae* were obtained from Sigma-Aldrich (Spain).

Other reagents used were of analytical grade and ultrapure water was used for preparing all the solutions.

2.2. Dry-cured ham processing and physico-chemical analyses

Hams were selected from animals reared according to routine practice guidelines of management and veterinary control. The diet contained lard as saturated fat and a premix of mineral and vitamins with 1.3 mg of riboflavin, 3.5 mg of pyridoxine and 10 mg of nicotinic acid per kg of feed.

Two series of paired hams, 36 hams for standard process (SP) and 50 hams for long process (LP), were selected from a commercial slaughterhouse at 24 h post-mortem (10–12 kg of weight for short process, 12–14 kg of weight for long process, and pH_{24} values ranging between 5.5 and 6.0). After 48 h at 2–3 °C every pair of ham was randomly assigned to five different nitrifying treatments according to the experimental design (Table 1a).

Hams were thoroughly rubbed with 10 g kg^{-1} of a mixture of NaCl and nitrifying agents (see Table 1b), and covered with salt for a period of 1 day per kg at 2–4 °C. Then, they were washed with water at 14 °C and hung for 2.5 months at 4 °C and 75–80% RH, placed in such a way that the two hams from the same carcass maintained the same location. Then, they were ripened at standard conditions of temperature (from 10 °C up to 24 °C) and relative humidity (from 80% down to 55%) during 6.5 and 17 months for SP and LP, respectively. Afterwards, they remained in cellar (10–12 °C) for 2.5 months.

At the end of the ripening period, dry-cured hams were dissected and *Gastrocnemius* muscle was trimmed of fat and connective tissue, ground, vacuum packed in aluminium foil bags and stored at −20 °C until analysis. Analyses were performed within 2 months.

Weight losses were reported all along the elaboration process. Chlorides and moisture were determined in triplicate by Near Infrared Technology (NIR) using the FoodScan® (FOSS®, Denmark) (Anderson, 2007) and residual nitrates and nitrites were determined according to ISO methods 3091 and 2918 (ISO 3091, 1975; ISO 2918, 1975) at the end of the process.

2.3. Determination of vitamins of the B group

Vitamin B1 (thiamine), vitamin B3 (nicotinamide), vitamin B2 (riboflavin) and vitamin B6 (pyridoxine) were extracted according to Ndaw, Bergaentzlé, Aoudé-Werner, and Hasselmann (2000). Briefly, 1 g of sample was added with 9 mL of 0.1 M HCl and maintained at 100 °C for 30 min in a water-bath. After cooling, 6 mL of 2.5 M sodium acetate and 1 mL of 10% (p/v) taka-diastase solution were added. Samples were incubated at 37 °C overnight and then centrifuged at 4 °C, and 4500 ×g for 5 min. Supernatant was collected and taken to 20 mL with ultrapure water.

Table 1a
Experimental design. distribution of paired hams in each treatment. Cross in the same row corresponds to a treatment comparison.

Treatment code				
Hi–N	Lo–N	Hi–Mix	Lo–Mix	Hi–Mix/Asc
X	X			
X		X		
	X		X	
		X	X	
		X		X

Table 1b
Treatments of hams according to the different concentrations of nitrifying salts added.

Treatment code	Composition of nitrifying salt
Hi–N	600 mg of KNO_3 kg^{-1}
Lo–N	150 mg of KNO_3 kg^{-1}
Hi–Mix	600 mg of KNO_3 kg^{-1} + 600 mg of $NaNO_2$ kg^{-1}
Lo–Mix	150 mg of KNO_3 kg^{-1} + 150 mg of $NaNO_2$ kg^{-1}
Hi–Mix/Asc	600 mg of KNO_3 kg^{-1} + 600 mg of $NaNO_2$ kg^{-1} + 500 mg of Na ascorbate kg^{-1}

Five mL of crude extract was treated as described by Gratacós-Cubarsí, Sárraga, Clariana, García Regueiro, and Castellari (2011) to determine vitamin B1, while other 5 mL was purified onto a Oasis® MCX cartridge (6 cm^3–150 mg, Waters Corp., Manchester, UK) to determine simultaneously vitamins B2, B3 and B6. HPLC-DAD/FLD analysis was performed using a Waters System equipped with a 717Plus autosampler, a 1525 binary HPLC pump, a 2996 Photodiode Array Detector and a 2475 Multi λ Fluorescence Detector (Waters Corp., Milford, MA, USA). The chromatographic separation was performed at room temperature on an Atlantis T3 (150 mm × 3.0 mm i.d., particle diameter 3 μm) column (Waters Corp., Milford, MA, USA) at a flow of 0.5 mL min^{-1}. A linear gradient elution between solvent A (0.2% H_3PO_4 20% w/v in methanol) and solvent B (0.2% H_3PO_4 20% w/v in water) was carried out by varying B % from 100% to 30% from 5 to 10 min.

Quantification of vitamin B3 was made by DAD, acquiring the signal at 260 nm whereas quantification of vitamin B2 and B6 was done by FLD (setting $\lambda_{EX} = 260$ and $\lambda_{EM} = 565$ for B2 and $\lambda_{EX} = 290$ and $\lambda_{EM} = 395$ for vitamin B6).

2.4. Determination of antioxidant enzyme activities

Sample was extracted according to the procedure of DeVore and Greene (1982). GSHPx activity was assessed by measuring the decrease of absorbance at 340 nm of the assay medium containing 0.5 units of GR, 1 mmoL L^{-1} GSH, 0.15 mmoL L^{-1} NADPH and 0.12 mmoL L^{-1} H_2O_2, recorded over 5 min. The activity was expressed as nmoL of NADPH oxidized per minute per g of tissue. SOD activity was measured according to the method of Marklund and Marklund (1974), based on the ability of SOD to inhibit the autoxidation of pyrogallol. The rate of autoxidation was determined by the absorbance measured at 420 nm. The enzyme activity was calculated according to a SOD standard curve (0 to 200 ng). One unit of enzyme activity was defined as the amount of the enzyme that inhibited the autoxidation of pyrogallol by 50%. Activity was expressed as units of SOD per g of tissue. CAT activity was determined by measuring the decrease in hydrogen peroxide at 240 nm for 5 min (Mei, Crum, & Decker, 1994). One unit of CAT activity was defined as the amount of CAT needed to decompose 1 μmoL H_2O_2 min^{-1}. Activity was expressed as μmoL of H_2O_2 decomposed per minute per g of tissue.

2.5. Measurement of lipid oxidation by the iron-induced TBA test

The iron-induced TBA test was a modification of the method of Kornbrust and Mavis (1980). Sample (3 g) was homogenised in 27 mL of 1.15% KCl. A 200 μL aliquot of the homogenate was mixed with 1 mL of 80 mM Tris/maleate buffer, pH 7.4, 400 μL of ascorbic acid and 400 μL of ferrous sulphate and incubated for different periods of time (0, 50, 100 and 150 min) in a 37 °C water bath with gentle stirring. Then, 4 mL of 26 mM TBA, 0.92 M TCA and 0.8 mM HCl were added and held for 15 min in boiling water. Samples were cooled and the absorbance was recorded at 532 nm vs. blanks. The