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Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: Typical microbiota, residual nitrate and nitrite and volatile profile

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ABSTRACT

Despite the number of studies that have focused on alternatives to nitrite in meat products, it has not been found yet a single compound that performs all its functions. Therefore, nitrate and nitrite are still common additives in the meat industry. However, to reduce nitrosamine formation, both ingoing amounts and residual levels must be controlled. Dry-fermented sausages were prepared with the maximum amounts of nitrate and nitrite allowed by the European Union, 25% and 50% reduction, and no nitrate/nitrite. The concentration of these additives significantly affected Gram-positive catalase-positive cocci, which numbers were 1 and 2 log cfu/g higher in the 50% reduction and control batches, respectively. A higher amount of volatiles derived from amino acid degradation and carbohydrate fermentation was detected, related to the microbiological changes. *Enterobacteriaceae* increased during fermentation at lower nitrate/nitrite concentrations. A relation was found between ingoing and residual nitrite, which was 3.5 fold higher when the maximum amount was used in comparison to the 50% reduction.

1. Introduction

Dry-fermented sausages are traditionally manufactured using nitrate and nitrite. These curing salts are added as preservatives due to the antimicrobial activity of nitrite, mainly related to the inhibition of the growth of *Clostridium botulinum* and its toxin production (Sebranek & Bacus, 2007). Furthermore, nitrite has some other technological roles, such as its influence on colour and flavour formation in cured meat products (Flores & Bermell, 1996; Marco, Navarro, & Flores, 2008; Noel, Briand, & Dumont, 1990; Olesen, Meyer, & Stahnke, 2004) and its contribution to the oxidative stability of lipids, which greatly influence aroma generation (Stahnke, 1995).

However, the involvement of nitrite in the formation of nitroso-compounds, such as carninogenic N-nitrosamines, makes the consumption of processed meats a matter of controversy (Corpet, 2011). Fresh red meat and processed meat have been associated with an increased risk of cancer, which would be related to haem iron. Among other mechanisms, haem iron would promote the synthesis of nitroso-compounds in the gastrointestinal tract; this endogenous production is caused by haem iron present in fresh meat (Cross, Pollock, & Binghamet, 2003), but it would be enhanced by the addition of nitrite to cured meats (Joosen et al., 2009).

In this context, the use of nitrate and nitrite in the food industry is strictly regulated. When the link between nitrite and N-nitrosamines was found in the early 1970s, a number of studies looking for possible substitutes were published (Pegg & Shahidi, 2000). The most recent alternatives proposed include the use of vegetable extracts (i.e. celery), natural antimicrobials (i.e. lactate, bacteriocins or direct addition of bioprotective lactic acid bacteria cultures) and a strict control of the manufacturing process. Nevertheless, up to date nitrite is still unique, since it has not been found a single compound that performs all its functions. On the other hand, certain strategies that can appear as more natural to consumers, such as the use of plant extracts, involve the indirect addition of nitrate and nitrite to the product (Sebranek & Bacus, 2007).

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The European Directive 2006/52/EC establishes a maximum amount of 150 mg/kg of each, nitrate and nitrite that may be added during the manufacture of dry-fermented sausages or 250 mg/kg of nitrate in long-cured products when no nitrite is added (European Parliament, 2006). In 2007, the Danish authorities communicated their intention to maintain a more restrictive regulation (i.e. a maximum nitrite addition of 100 mg/kg to fermented salami) as they considered that the application of higher values could result in a significant rise in the intake of nitrite and thereby nitrosamines (European Commission, 2010). Considering this background, Regulation N° 1129/2011 (European Commission, 2011) mentioned the possibility to reduce in a future the current maximum inputs added to all meat products in the European Union (EU).

On the other hand, the interest of the consumers for organic meat products has increased during the last years as they are considered healthier and more natural. Regulation N° 889/2008 establishes the list of permitted additives for the production of certain organic foods within the EU (European Commission, 2008). This regulation sets an indicative ingoing amount of 80 mg/kg and a maximum residual amount of 50 mg/kg of nitrate and nitrite for organic meat products, which represents about a 50% reduction of the amount of these additives in comparison to non-organic ones.

In view of a possible change of the current European regulations, it is necessary to assess the effect of reducing the concentration of nitrate and nitrite not only in the growth of pathogens but also on other parameters that may affect the stability and quality of the product. For this purpose, *chorizo*, a traditional high-acid (pH < 5.5) dry-fermented sausage highly consumed in Spain and Portugal, was selected. We have analysed the typical microbiota (lactic acid bacteria, Gram-positive catalase-positive cocci, and also *Enterobacteriaceae*), the volatile profile and the residual levels of nitrate/nitrite in *chorizo* manufactured with the maximum amount currently allowed by the EU regulations, and with a 25% and 50% reduction.

2. Materials and methods

2.1. Sausage manufacture

Sausages were manufactured at the Research and Technology Food and Agriculture Institute (IRTA, Monells, Spain) by grinding separately lean pork (90%) (minced with a 25 mm plate) and pork belly (10%) (minced with a 12 mm plate), which were then mixed with common ingredients at 0 °C for 2 min under vacuum conditions: NaCl (2.4%), distilled water (2%), sweet paprika (1.2%), dextrose (0.7%), sodium acid pyrophosphate (0.15%) and a commercial starter of *Pediococcus pentosaceus* and *Staphylococcus xylosus* (Clerici Sacco International, Cadorago, Italy). The batter was then divided into four batches which were added with different concentrations of nitrate/nitrite dissolved in water (1% of the formula): 1) 150 mg/kg KNO_3 and 150 mg/kg $NaNO_2$ (high nitrate/nitrite batch, HN); 2) 112.5 mg/kg KNO_3 and 112.5 mg/kg $NaNO_2$ (25% reduction: medium nitrate/nitrite batch, MN); 3) 75 mg/kg KNO_3 and 75 mg/kg $NaNO_2$ (50% reduction: low nitrate/nitrite batch, LN) and 4) a control batch with no nitrate/nitrite (C).

All batches were mixed for 3 additional minutes under vacuum and then stuffed into 80 mm diameter collagen casings (Fibran, Sant Joan de les Abadesses, Spain) using a SIA Junior vacuum stuffer (SIA, Palau de Plegamans, Spain), resulting in sausages of *ca.* 400 g, which were dipped for few seconds in water containing 10 g/l of pimaricin, to prevent fungal growth. The sausages were hung vertically and allowed to reach 14–16 °C for 24 h at 70–80% relative humidity (RH). Afterwards, they were incubated at 24–26 °C and 90% RH for fermentation until pH decreased to 5.0, and dried at 12 °C and 75–80% RH for 24 days.

Sausages were sampled for microbial counts, pH, a_w and nitrate/nitrite determinations at days 0, 3, 14 and 27 of ripening. Volatile analysis was performed at the end of ripening.

2.2. Physico-chemical analysis

Measurement of pH was done in a homogenate prepared with an aliquot of sausage (4 g) and distilled water (10 ml), using a Crison 2001 pH meter (Crison Instruments, Barcelona, Spain). Water activity (a_w) values were obtained at 25 °C from a 2 mm thick sausage slice using a dew point hygrometer Decagon CX-1 (Decagon Devices, Pullman, WA).

2.3. Microbiological analysis

Ten gram sausage samples were homogenized with 50 ml of saline solution (0.85% NaCl) for 2 min in a Stomacher 400 (Colworth, London, UK). Serial ten-fold dilutions were prepared from this homogenate.

Total viable counts (TVC) were determined on Plate Count Agar (PCA) (Pronadisa, Madrid, Spain) with 1% NaCl according to the recommendations by Dainty, Shaw, Boer, and Scheps (1975), and incubated at 32 °C for 48 h; lactic acid bacteria (LAB) were plated on double layer de Mann, Rogosa and Sharpe (MRS) agar at pH 5.5 and incubated at 32 °C for 48 h; Gram-positive catalase-positive cocci (GCC+) were enumerated on Manitol Salt Agar (MSA) (Pronadisa) at 32 °C for 48 h, and *Enterobacteriaceae* on double layer Violet Red Bile Glucose Agar (VRBG) (Pronadisa) incubated at 37 °C for 24 h. For the enumeration of sulphite-reducing clostridia, 1 ml of the corresponding dilution was heated at 80 °C for 10 min and then inoculated in tubes containing Sulphite Polymyxin Sulfadiazine (SPS) melted agar (Pronadisa). To maintain anaerobic conditions, an overlay of sterile paraffin was added before incubating the tubes at 37 °C for 24 h. The results were expressed as cfu/g of sausage.

2.4. Determination of residual nitrate and nitrite

Residual nitrate and nitrite were determined using Flow Injection Analysis (FIA). Nitrite was determined according to the procedure described by Ruiz-Capillas, Aller-Guiote, and Jiménez-Colmenero (2007). Briefly, nitrite reacts with sulphanilamide to form a diazonium salt which is added to N-(1-Naphthyl)-ethylenediamine dihydrochloride (NED) to yield an azo dye compound, which absorbance is spectrophotometrically determined at 540 nm. The extract used for the analysis was prepared from 10 g of the sample according to the AOAC method (AOAC, 1990), with a final volume of 250 ml. This extract was injected into the FIA manifold to measure residual nitrite. Nitrate was determined after its reduction to nitrite using a cadmium reductor (FOSS Tecator, Sweden) placed in the FIA system (Ruiz-Capillas, Aller-Guiote, Carballo, & Jiménez Colmenero, 2006). Nitrate content was estimated by the difference between the nitrite content after the reducing process and the residual nitrite. Standard solutions with concentrations from 0.125 to 4 mg/l of nitrite and nitrate were prepared from a stock solution of 1000 mg/l.

2.5. Volatile compound analysis

The extraction of the headspace volatile compounds was done using a solid phase microextraction (SPME) device containing a fused silica fibre (10 mm length) coated with an 85 μm carboxen/polydimethylsiloxane (CAR/PDMS) fibre (Supelco, Bellefonte, PA).

Ripened samples were ground with a commercial grinder and 4 g were transferred to a 15 ml vial, sealed with a PTFE faced silicone septum (Supelco). The vial was left for 30 min in a