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A study on the toxigenesis by *Clostridium botulinum* in nitrate and nitrite-reduced dry fermented sausages

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ABSTRACT

Nitrite has been traditionally used to control *Clostridium botulinum* in cured meat products. However, in the case of dry fermented sausages, environmental factors such as pH, a_w and the competitive microbiota may exert a more relevant role than nitrite in the inhibition of the growth and toxin production by *C. botulinum*. In this challenge test study, two varieties of Mediterranean dry sausages (*salchichón* and *fuet*) were inoculated with spores of *C. botulinum* Group I (proteolytic) and *C. botulinum* Group II (nonproteolytic). Sausages were prepared with 150 mg/kg of $NaNO_3$ and 150 mg/kg of $NaNO_2$ (maximum ingoing amounts allowed by the European Union regulation), with a 25% and 50% reduction, and without nitrate/nitrite. The initial pH in both products was 5.6, and decreased to values below 5.0 in *salchichón* and to 5.2 in *fuet*. Lactic acid bacteria counts reached 8–9 log cfu/g after fermentation. The a_w decreased from initial values of 0.96 to about 0.88–0.90 at the end of ripening. Botulinum neurotoxin was not detected in any of the sausages, including those manufactured without nitrate and nitrite. Despite the environmental conditions were within the range for germination and growth of *C. botulinum* Group I during the first 8 days of the ripening process in *fuet* and 10–12 days in *salchichón*, acidity, a_w and incubation temperature combined to inhibit the production of toxin, independently of the concentration of curing agents. Although decreasing or even removing nitrate/nitrite from the formula did not compromise safety regarding *C. botulinum* in the conditions tested in this study, their antimicrobial role should not be underestimated in the case that other hurdles could fail or other ripening conditions were used, and also considering the effect of nitrite on other pathogens.

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1. Introduction

Mediterranean-style dry sausages are based on a combination of meat curing and drying, together with fermentation in most cases. Their long shelf life is a consequence of successive factors or hurdles, such as the addition of nitrite and salt, the low pH due to the growth of lactic acid bacteria (LAB), a low redox potential and intermediate water activity (a_w) (Leistner, 1995).

Nitrate and nitrite are commonly used additives in cured meats because of their technological contribution to the oxidative stability of lipids and to the typical flavour and colour development, which can be achieved with the addition of approximately 40–50 mg/kg of nitrite (Sebranek and Bacus, 2007). Moreover, a key role on the inhibition of the Gram-positive spore-forming anaerobic bacteria *Clostridium botulinum* has been attributed to nitrite. Such inhibition is the result of the interaction between nitric oxide derived from nitrite and clostridial iron–sulphur proteins, such as ferredoxin and ferredoxin-pyruvate oxidoreductase, resulting in a rapid decrease of intracellular ATP and pyruvate accumulation (Tompkin, 2005; Woods and Wood, 1982).

Raw sausage mixtures may contain significant numbers of spore-forming bacteria, with spices being a major source (ICMSF, 2005). However, germination and growth, and also toxin production, will depend on environmental factors such as pH and a_w. The role of nitrite in the inhibition of *C. botulinum* in cured meat products remains controversial. Some authors state that toxin formation is hardly probable, especially in European-type fermented sausages due to their low pH and a_w (Lücke, 2000), while other studies consider that nitrite appears to be necessary, especially in traditional dry fermented sausages with a mild pH drop (EFSA, 2003).

Four physiologically distinct groups of *C. botulinum* have been described, with Group I (proteolytic) and Group II (nonproteolytic) responsible for virtually all cases of foodborne botulism (Peck, 2006). *C. botulinum* Group I produce botulinum neurotoxin (BoNT) types A, B and/or F; their growth is inhibited at 10 °C or below, at pH ≤ 4.6, in the presence of 10% NaCl or at a_w below 0.94. *C. botulinum* Group II produce BoNT types B, E and F, they are able to grow and form toxin at 3 °C, but they do not grow or form neurotoxin at pH < 5 and at NaCl concentration above 5%, and at a_w below 0.97 (Johnson, 2013; Lund and Peck, 2000).

Despite the relevant technological and safety functions of nitrite, there is great concern regarding its role in the formation of N-nitrosamines

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(Pegg and Shahidi, 2000). Balancing the risks and the benefits of using nitrate and nitrite, EU Regulation no. 1129/2011 establishes the maximum amount of these additives that may be added during manufacturing of cured meat products (European Comission, 2011), which for dry fermented sausages is 150 mg/kg of nitrate and 150 mg/kg of nitrite, or for long ripening sausages, 250 mg/kg of nitrate if nitrite is not added. However, consumer demands for healthier products, with less additives, together with the request by some EU member states to maintain its more restrictive regulations, could result in a future revision of the current levels of nitrate and nitrite allowed in the manufacture of meat products. Even if the presence of spores of *C. botulinum* in meat is generally low (<1–10 spores/kg) (Dodds, 1993), and their growth is naturally inhibited by the different hurdles in dry sausages, the safety of a product has to be re-evaluated when modifying the formulation, the process or storage conditions (EFSA, 2005). It has been reported that very low amounts of preformed toxin in foods i.e. 30–100 ng result in a severe and deadly intoxication (Peck, 2009).

The aim of this work was to investigate toxin production by *C. botulinum* Group I and *C. botulinum* Group II in two Mediterranean-style dry sausages with different pH values and manufactured with different concentrations of nitrate and nitrite.

2. Materials and methods

2.1. Preparation of spores

Two different cocktails of *C. botulinum* spores were prepared. Cocktail 1 contained spores of three Group I type A strains (62A, Eyemouth and ATCC 3502); cocktail 2 contained spores of six Group II strains: three type B strains (Eklund 2B, CDC 3875 and Kapchunka B2) and three type E strains (Beluga, Dolman VH and CDC 7854). The strains chosen had been isolated from a range of different materials, on different continents over many decades (Table 1).

Stock spore suspensions that had been produced in a two-phase medium of water over cooked meat agar as described by Peck et al. (1992) were used. These spores had been stored at 2 °C aerobically in water. Before use, the spore preparations were enumerated and tested for toxin production and proteolytic activity. All the spore suspensions were observed microscopically to check if they still contained a high proportion of phase bright spores. Each suspension was enumerated by growth on pre-reduced Peptone Yeast Extract Glucose Starch agar (PYGS, Oxoid) after incubation at 30 °C for 3 days under H_2/CO_2 (90:10). All suspensions contained more than 10^8 viable spores/ml. To determine any aerobic contamination, spores were aerobically incubated on Tryptone Soya Agar plates (TSA, Oxoid) at 30 °C for 2 days. Each strain was also tested for its ability to produce toxin in standard culture media pH 6.8 incubated at 30 °C for 24 h, using the same ELISA method as was used for the sausage samples (Table 1). Proteolytic activity was assessed by the ability to digest casein on pre-reduced Reinforced Clostridial Medium (RCM) (Oxoid, Basingstoke, UK) plates containing 5% (*w*/*v*) skim milk powder, which were incubated at 30 °C for 3 days under H_2/CO_2 (90:10).

To prepare the inocula, the spores were diluted in 0.85% saline solution and mixed to give separate cocktails for *C. botulinum* Group I and *C. botulinum* Group II each with a final concentration of 7 log spores/ml comprised of equal numbers of spores of each constituent strain.

2.2. Preparation of the starter cultures

For *salchichón*, a combination of *Lactobacillus plantarum* CECT 220, *Staphylococcus xylosus* CECT 237 and *Staphylococcus carnosus* CECT 4491 was used as starter cultures. All the strains were obtained from the Spanish Collection of Type Cultures (CECT, Valencia, Spain). The two staphylococci and the lactobacilli were grown at 37 °C in Tryptone Soy Broth (TSB) (Pronadisa, Madrid, Spain) and in pH 5.7 de Mann, Rogosa and Sharpe (MRS) broth (Pronadisa), respectively, to reach the stationary growth phase.

A commercial *Penicillium candidum* culture (Ascott Smallholding Supplies, Newton Abbot, UK) was used for surface inoculation of *fuet*.

2.3. Preparation of sausages

Two traditional varieties of Mediterranean-style dry sausages were selected, *salchichón* and *fuet*. Both products are based on lean pork (or pork and beef mixtures) together with pork back fat and pepper as characteristic spice. *Salchichón* reaches a lower pH due to lactic acid fermentation, which is promoted by the addition of sugars and the use of an initial ripening temperature above 20 °C. *Fuet* is characterized by a higher pH resulting from the control of fermentation at a lower initial temperature (usually below 15 °C) and avoiding the addition of lactic acid starters and sugars. Furthermore, *fuet* usually has a smaller diameter than *salchichón* and presents a typical white coating due to fungal growth.

In our study, sausages were manufactured with a mixture of lean pork and pork back fat prepared in a mincer equipped with a 5 mm hole diameter plate. The mixture was divided into four batches to which different nitrate/nitrite concentrations were added: batch 1) 150 mg/kg of $NaNO_3$ and 150 mg/kg of $NaNO_2$ (high nitrate/nitrite batch, HN), at present this is the maximum amount allowed by the EU for products to be ripened for less than 30 days; batch 2) 112.5 mg/kg of $NaNO_3$ and 112.5 mg/kg of $NaNO_2$ (25% reduction: medium nitrate/nitrite batch, MN); batch 3) 75 mg/kg of $NaNO_3$ and 75 mg/kg of $NaNO_2$ (50% reduction: low nitrate/nitrite batch, LN); and batch 4) control sausages with no nitrate/nitrite added (C). Once the batches were obtained, both *C. botulinum* Group I and *C. botulinum* Group II spore cocktails were added to each batch to reach a concentration of 4 log spores/g, followed by manual mixing.

Afterwards, the formula of *salchichón* was completed by adding water, NaCl, dextrose, lactose and ground black pepper (Table 2). The starter culture was added in a proportion 1:1:1 to reach an approximate

Table 1
Clostridium botulinum strains included in the study.

Strain	Group	Toxin type	Toxin titre (ng/ml)[a]	Initial isolation	Present source
ATCC 3502	I	A	>100	California, USA, early 1920s	CAMR, 2000
Eyemouth	I	A	10	?	TRS, 1993
62A	I	A	>100	Canned food, USA, pre1948,	IFR, 1993
Eklund 2B	II	B	>25	Pacific sediment, USA, 1965	UR, 1983
CDC 3875	II	B	>25	Human stool, Iceland, 1981	CDC, 1987
Kapchunka B2	II	B	10	Kapchunka, USA, 1981	NFPA, 1993
Beluga	II	E	>100	Fermented Beluga whale, USA, 1950	UR, 1981
Dolman VH	II	E	>100	Pickled herring, Canada, 1949	CDC, 1987
CDC 7854	II	E	>100	Fish, Egypt, 1991	CDC, 1993

CAMR = Centre for Applied Microbiology and Research; TRS = L. Taylor, Torry Research Station; IFR = T. Roberts, Institute of Food Research, UK; UR = J. Crowther, Unilever Research, UK; CDC = C. Hatheway, Centres for Disease Control, USA; NFPA = V. Scott, National Food Processors Association, USA.

[a] Toxin concentration of culture supernatant was measured by ELISA against dilutions of toxin standard (Metabiologics Inc., Wisconsin, USA).

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