Food Research International 101 (2017) 82–87

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Effect of nitrate and nitrite on *Listeria* and selected spoilage bacteria inoculated in dry-cured ham

Xavier F. Hospital[a], Eva Hierro[a,*], Jacint Arnau[b], José Carballo[c], Juan S. Aguirre[a], Marta Gratacós-Cubarsí[b], Manuela Fernández[a]

[a] Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain
[b] Institut de Recerca i Tecnologia Agroalimentàries (IRTA), 17121 Monells, Spain
[c] Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN-CSIC), 28040 Madrid, Spain

ARTICLE INFO

Keywords:
Dry-cured ham
Nitrate/nitrite
Listeria innocua
Serratia liquefaciens
Proteus vulgaris

ABSTRACT

The effect of nitrate and the combination of nitrate/nitrite on *Listeria innocua* (as surrogate of *Listeria monocytogenes*). And two selected spoilage microorganisms (*Proteus vulgaris* and *Serratia liquefaciens*) was studied in dry-cured ham. Hams were manufactured with different concentrations of curing agents: KNO_3 (600 and 150 mg/kg) alone or in combination with $NaNO_2$ (600 and 150 mg/kg). The addition of 500 mg/kg of sodium ascorbate was also evaluated in a batch with 600 mg/kg of nitrate and nitrite. The target microorganisms were inoculated by injection in *semimembranosus*, *biceps femoris* and in the shank, prior to curing. *P. vulgaris* and *S. liquefaciens* were controlled by temperature and a_w, respectively, and no effect of nitrate/nitrite was observed. The presence of nitrite in the curing mix reduced *L. innocua* in *semimembranosus*, which population was 1.5 log cfu/g lower at the end of resting ($p < 0.05$), while at the end of the process it was more frequently detected in the no- and low-nitrite added hams. None of the treatments was able to control *Listeria* in deeper areas of ham. The addition of sodium ascorbate to the curing mix containing the highest amount of nitrate and nitrite did not show any effect on the microorganisms studied.

1. Introduction

Curing of whole pieces of meat has a long tradition in different regions of the world. Among such traditional products there are dry-cured meats, in which the addition of salt and often nitrate and/or nitrite, followed by drying, result in stable and highly appreciated products, such as non-smoked dry-cured hams, of which Spain, Italy and France are the main producers in Europe (Estévez, Ventanas, Morcuende, & Ventanas, 2015).

Since ancient times, nitrate impurities present in salt had contributed to meat preservation and sensory quality, although their role was not scientifically demonstrated until the study of the chemistry of nitrite started at the end of the nineteenth century (Pegg & Shahidi, 2000). Nitrate and nitrite can be used alone or in combination in dry-cured ham. Nitrate acts as a reservoir of nitrite, which is formed by the action of nitrate-reductase enzymes present in some microorganisms (*i.e. Staphylococcus* spp.). Nitrite is a highly reactive compound that contributes to the typical colour and flavour, reduces oxidative rancidity (Honikel, 2008; Sebranek & Bacus, 2007; Skibsted, 2011) and exerts an important role on the safety and quality of these products related to the inhibition of spoilage and pathogenic bacteria such as *Clostridium botulinum* (EFSA, 2003; Sebranek & Bacus, 2007) and *Listeria* sp. (Hospital, Hierro, & Fernández, 2012).

The use of nitrifying salts in cured meats began to be controversial in the 1960s due to their involvement in the formation of *N*-nitrosamines, which are potent carcinogenic compounds (Pegg & Shahidi, 2000). Reducing agents, such as ascorbate or erythorbate are added to meat products to stabilize colour and to diminish nitrosamine formation (Chow & Hong, 2002; Hu & Chen, 2010). In any case, the amount of both added and residual nitrate/nitrite must be controlled in meat products. For this reason, the use of these additives is regulated by the food safety authorities. The European Union, by means of Regulation 1129/2011, sets maximum residual amounts of 250 mg/kg for nitrate and 100 mg/kg for nitrite in dry-cured hams at the end of the process (European Commission, 2011). These levels could be revised as the European authorities are considering the possibility of reducing the amount of nitrate and nitrite used in meat products in the future (Food Chain Evaluation Consortium, 2016). However, any reduction in the use of nitrifying salts must achieve a balance between microbiological and toxicological safety, and, obviously, quality.

* Corresponding author.
E-mail address: hierro@ucm.es (E. Hierro).

http://dx.doi.org/10.1016/j.foodres.2017.08.039
Received 26 May 2017; Received in revised form 17 July 2017; Accepted 16 August 2017
Available online 18 August 2017

In the production of dry-cured hams, nitrate/nitrite is generally included in the curing mix. This is typically the case of Spanish hams. Nitrate and nitrite, together with a_w, contribute to the safety and stability of the product. A decrease of the amounts of these additives in order to reduce residual levels could affect ham microbiota, increasing the growth of spoilage microorganisms and allowing survival/growth of pathogens, if present in the green ham. Microorganisms can reach the surface of fresh hams from different environmental sources, but can also get into deeper areas due to the presence of cracks in the musculature, or during slaughter and cutting through faecal contamination transmitted by the circulatory flow (Autio et al., 2000; Losantos, Sanabria, Cornejo, & Carrascosa, 2000; Troeger & Woltersdorf, 1986). Furthermore, salt-tolerant microorganisms present in brines can also penetrate into the pieces (Cordero & Zumalacárregui, 2000; Rodríguez, Martín, & Nuñez, 2001).

Proteus vulgaris and *Serratia liquefaciens* are among the most important microorganisms responsible for spoilage of dry-cured hams, such as bone taint or deep spoilage (García, Martín, Timón, & Córdoba, 2000; Marín, Carrascosa, & Cornejo, 1996; Martín et al., 2008; Paarup, Nieto, Peláez, & Reguera, 1999). *S. liquefaciens* is among the most common microorganisms found on work surfaces in the meat processing industry (Stiles & Ng, 1981). Although bone taint is detected in the finished product, the spoilage itself begins during the first stages of the process (Bersani, Cattaneo, Cantoni, & Balzetti, 1984; Córdoba et al., 1994). The occurrence of this defect has been related to inadequate chilling during cutting, transport or salting and resting (post-salting) stages (Marín et al., 1996). In addition, faulty salting could also favour microbial growth at the higher temperatures used during drying; in this case, the decrease of a_w to inhibitory levels would be slowed down in some parts due to low water losses and low salt uptake or diffusion (García et al., 2000; Marín et al., 1996; Martín et al., 2008).

A significant prevalence of *Listeria monocytogenes* has been detected in live pigs, mainly in tongue, tonsils and also in the intestinal content (Hellström et al., 2010; Prencipe et al., 2012). Therefore, asymptomatic animals may be a direct source of cross-contamination of meat during handling for the production of hams. The raw materials can also contaminate equipment and environment, and due to the ability of *Listeria* spp. to form biofilms and adhere to materials, it is highly persistent and ubiquitous in food processing facilities (Moretro & Langsrud, 2004). In a study throughout the Parma ham processing chain, Prencipe et al. (2012) found *L. monocytogenes* in 3% of pig carcasses, 12.5% in raw hams and 2% in the final product. For their part, Giovannini et al. (2007) reported a prevalence of 4% in dry-cured Parma and San Daniele hams. This organism is able to grow in a wide range of temperature and pH, and it tolerates a_w 0.90–0.92 and 16% NaCl concentration (European Union, 2008; FSAI, 2005); thus, if present in green hams, the processing conditions would allow its growth, at least in some stages of the process.

As previously mentioned, dry-cured meats are considered safe and stable products, due to the different hurdles acting together. However, some outbreaks have been reported associated to dry-cured ham consumption (González-Hevia, Gutiérrez, & Mendoza, 1996; Lyytikäinen et al., 2000; Untermann & Müller, 1992), and bone taint can be the cause of great economical losses in these highly valued products.

The aim of the present 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 on the survival of *Listeria* sp., *S. liquefaciens* and *P. vulgaris*.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

All the strains used in these experiments were obtained from the Spanish Collection of Type Cultures (CECT, Valencia, Spain): *Serratia liquefaciens* CECT 483 T and *Proteus vulgaris* CECT 4077, and *Listeria innocua* (CECT 910 and CECT 4030). *L. innocua* was used in this study since it is often regarded as the non-pathogenic variant of *L. monocytogenes* and it is a useful surrogate in a variety of treatments, including radiation, heat and lactic acid, sodium chloride, and nitrite addition (Kamat & Nair, 1996). The use of surrogates in challenge studies is a common practice when it is not possible to introduce pathogens into a food processing facility (IFT/FDA, 2003).

Stock cultures of each organism were prepared and maintained in Tryptone Soy Broth (TSB; Pronadisa, Madrid, Spain) with 15% glycerol at − 20 °C. To prepare the inocula, a loop of frozen culture was transferred to 9 ml of TSB, then incubated at 32 °C (*P. vulgaris* and *S. liquefaciens*) or 37 °C (*L. innocua*) for 24 h; subsequently, a 20 µl inoculum of the grown cultures was transferred to 10 ml of TSB and incubated under the same conditions. Afterwards, each microorganism was plated on Tryptone Soy Agar (TSA; Pronadisa) and incubated under the same conditions. For each organism, one colony was transferred into 10 ml of TSB and incubated at 32 °C/37 °C to reach the stationary growth phase. Independent inocula were prepared for each species at a concentration of 10^5 cfu/ml. The two strains of *L. innocua* were mixed in equal concentrations to obtain a single inoculum.

2.2. Preparation and inoculation of hams

Sixty-eight hams were selected at 24 h post-mortem (10–12 kg of weight and pH_{24} values ranging between 5.5 and 6.0). Measurements of pH_{24} were carried out in the *semimembranosus* muscle of the green ham with a portable pH-meter Crison 507 (Crison Instruments, Barcelona, Spain). The hams were obtained from 34 animals and the two green hams from each carcass were assigned to different treatments in order to exclude the variable “animal” (individual) in the comparison of the results. The experimental design is shown in Tables 1 and 2. The treatments were balanced by pH_{24} and ham weight.

After 48 h at 2–3 °C, and prior to curing, hams were inoculated with 200 µl of the corresponding microbial culture in different parts of the piece (two points per microorganism): *semimembranosus*, *biceps femoris* and shank (Fig. 1). For this purpose, syringes with 1.2 × 40 mm hypodermic needles were used. In order to reduce the flow-back of the inoculum, each inoculation point was manually massaged for 10–15 s immediately after puncture. All hams were inoculated with *L. innocua*, while spoilage bacteria (*P. vulgaris* and *S. liquefaciens*) were only inoculated in 34 hams, which were analyzed at the end of resting, to prevent deterioration. The total ripening period was 12 months.

For curing, hams were thoroughly rubbed with 10 g/kg of a mixture of NaCl and different concentrations of KNO_3 and $NaNO_2$ (Table 1). An additional batch including sodium ascorbate was also prepared. The maximum amounts used in this study were similar to those used in a previous work by Gratacós-Cubarsí et al. (2013), yielding a mean residual nitrate and nitrite content lower than the maximum levels allowed by Regulation (EU) 1129/2011. The hams were then covered with humid salt (4% water content) for 1 day/kg at 2–4 °C, and, afterwards, washed with water and hung for 2.5 months at 2–4 °C and 75–80% relative humidity (RH). At this point (end of the resting stage), 34 hams were sampled. The remaining hams were ripened at increasing temperature (from 10 to 24 °C) and decreasing RH (from 75 to 55%) during 6.5 months. Finally, they were kept in a cellar at 14–16 °C and

Table 1
Treatments and composition of the curing mix.

Treatment	Curing mix
Hi-N (high nitrate)	600 mg/kg KNO_3
Lo-N (low nitrate)	150 mg/kg KNO_3
Hi-Mix (high nitrate-nitrite)	600 mg/kg KNO_3 + 600 mg/kg $NaNO_2$
Lo-Mix (low nitrate-nitrite)	150 mg/kg KNO_3 + 150 mg/kg $NaNO_2$
Hi-Mix/Asc (high nitrate-nitrite with ascorbate)	600 mg/kg KNO_3 + 600 mg/kg $NaNO_2$ + 500 mg/kg Na ascorbate