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Effect of *Penicillium nalgiovense* as protective culture in processing of dry-fermented sausage "salchichón"

Victoria Bernáldez [a], Juan J. Córdoba [a], Mar Rodríguez [a], Mirian Cordero [a], Luis Polo [b], Alicia Rodríguez [a,*]

[a] Higiene y Seguridad Alimentaria, Facultad de Veterinaria, Universidad de Extremadura, Avda. de la Universidad, s/n. 10003, Cáceres, Spain
[b] Mataderos Industriales Soler S.A. (Prolongo)/Frigoríficos Andaluces de Conservas de Carne S.A. (FACCSA), Plaza Prolongo 1 – 2, 29580 Cártama Estación, Málaga, Spain

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ABSTRACT

In this work the implantation of a protective culture of *Penicillium nalgiovense* on commercial dry-fermented sausages "salchichón" and its effect over presence of mycotoxin-producing moulds belonging to contamination origin was evaluated. In addition, the suitability of real-time quantitative PCR (qPCR) as a rapid and sensitive method to test implantation of protective culture throughout the "salchichón" processing was also tested. Dry-fermented sausages "salchichón" inoculated with a non-toxigenic protective *P. nalgiovense* and subjected to three different commercial ripening processes were analysed. At first, ability of *P. nalgiovense* strain to avoid growth of an ocratoxin A (OTA)-producing strain and its mycotoxin production in a controlled model system was demonstrated. *P. nalgiovense* was quantified by a qPCR designed on the basis of the ITS region and values higher than 10^6 ufc/cm^2 in both inoculated and non-inoculated "salchichón" were obtained. This technique should be considered a good tool to verify the implantation of protective culture of *P. nalgiovense*. Producing moulds of aflatoxins, OTA, patulin, sterigmatocystin and verrucosidin and the corresponding mycotoxins were not detected in any dry-fermented sausages tested, including those non-inoculated ones. Thus, presence of *P. nalgiovense* is inhibiting growth of toxigenic moulds. Utilization of a non-toxigenic fungal protective culture in dry-fermented sausage "salchichón" processing should be considered as a good tool in the preventive programmes to avoid growth of toxigenic moulds.

1. Introduction

A rich variety of fermented meat sausages is produced in Spain (López-Díaz, Santos, García-López, & Otero, 2001). The environmental conditions in the manufacturing rooms for sausage production are very suitable for mould growth on the surface of products (Comi, Orlic, Redzepovic, Urso, & Iacumin, 2004; Mizakovà, Pipovà, & Turek, 2002). Some mould strains can produce undesirable effects on quality of these products associated with off-flavours, colour of conidia and floccose mycelium on the casing (Ludemann, Greco, Rodríguez, Basílico, & Pardo, 2010), but overall some strains may produce toxicological problems for consumers due to mycotoxin production (Battilani et al., 2007; Iacumin et al., 2009). The presence of mycotoxins in the meat products or on the sausage casing is certainly undesirable, since they are secondary metabolites with relevant toxics effects on consumer health. Among these compounds, aflatoxins, ochratoxin A (OTA), patulin, sterigmatocystin and verrucosidin are mycotoxins extremely toxic with carcinogenic and teratogenic effects (Beretta, Gaiaschi, Galli, & Restani, 2000; European Mycotoxins Awareness, 2011; Knaus et al., 1994; Mayer, Bagnara, Färber, & Geisen, 2003; Schmidt-Heydt, Abdel-Hadi, Magan, & Geisen, 2009). All of these compounds can be produced by different species belonging to *Aspergillus*, *Penicillium* and *Emericella* genera (Bogs, Battilani, & Geisen, 2006; Frisvad, Smedsgaard, Larsen, & Samson, 2004; Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2011; Mayer et al., 2003; Niessen, 2007; Rank et al., 2011; Sant'Ana et al., 2010; Schmidt-Heydt et al., 2009) when the appropriate ecological conditions in some foods such as dry-fermented sausage "salchichón" are found.

The application of protective non toxigenic selected moulds could avoid that dry-fermented sausages "salchichón" were spontaneously colonized by uncontrolled and heterogeneous fungal population where toxigenic strains may be present (Ludemann et al., 2010). For this reason, mould strains used as protective cultures during the processing to ensure the safety of the final

* Corresponding author. Tel.: +34 927 257 125; fax: +34 927 257 110.
E-mail address: aliciarj@unex.es (A. Rodríguez).
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product should be carefully selected (Comi et al., 2004; López-Díaz et al., 2001).

Non-toxigenic commercial *Penicillium nalgiovense* and *Penicillium chrysogenum* species are used as protective starter cultures in the meat industry on the casing of dry-fermented sausages (Leistner, 1990; Papagianni & Papamichael, 2007). It is assumed that these protective cultures avoid growth of uncontrolled fungal contamination during the drying and fermenting processes but this fact has not been demonstrated yet. It is necessary to evaluate implantation of these commercial protective cultures as well as presence of toxigenic moulds throughout usual commercial ripening process where contamination of products with undesirable moulds is possible. In addition, accumulation of mycotoxins and effect of these protective cultures against toxigenic moulds should be also evaluated. Due to difficulties of inoculating toxigenic moulds in the meat industry, effect of the protective culture should be first tested in controlled conditions in a dry-cured meat product (dry-fermented sausage "salchichón") model. Since it has been demonstrated that OTA-producing strains is the most serious concern in dry-fermented sausages (Iacumin et al., 2009), it could be studied effect of protective culture against OTA producers in a controlled inoculated dry-fermented sausage model.

To quantify the implantation of protective non-toxigenic culture moulds, rapid and quantitative techniques such as real-time quantitative PCR (qPCR) could be used. The qPCR is a specific and highly sensitive technique, since the mould DNA target can be detected in complex mixtures (González-Salgado, Patiño, Gil-Serna, Vázquez, & González-Jaén, 2009; Mateo, Gil-Serna, Patiño, & Jiménez, 2011). To date, several qPCR assays have been developed to quantify mycotoxin-producing moulds in foods using as target toxin biosynthesis genes, constitutive genes or multi-copy sequences (Gil-Serna, Gónzalez-Salgado, Gónzalez-Jaén, Vázquez, & Patiño, 2009; Mayer et al., 2003; Passone, Rosso, Ciancio, & Etcheverry, 2010; Sardiñas, Vázquez, Gil-Serna, Gónzalez-Jaén, & Patiño, 2011; Schmidt-Heydt et al., 2009). In this way, the last two targets could be also used for designing a qPCR method to quantify non-toxigenic protective culture moulds in foods. This methodology could be very useful as a rapid and sensitive tool for the verification of protective cultures in the HACCP programs.

The purpose of this work was to evaluate the implantation of a protective culture of *P. nalgiovense* on commercial dry-fermented sausages "salchichón" and its effect over presence of mycotoxin-producing moulds belongs to contamination origin. The effect of protective *P. nalgiovense* was first tested in a controlled dry-fermented sausage model inoculated with an OTA-producing strain. The suitability of qPCR as a rapid and sensitive method to test implantation of protective culture over dry-fermented sausage "salchichón" was also evaluated. In addition, the accumulation of mycotoxins in "salchichón" was analysed.

2. Material and methods

2.1. Sampling process

2.1.1. Controlled inoculated dry-fermented sausage model

To test the effectiveness of a protective commercial *P. nalgiovense* TEXEL® PN1 from Danisco (Niebüll, Germany) against an OTA-producing mould, slices of non-sterile commercial dry-fermented "salchichón" showing pH 5.36 ± 0.05 and water activity 0.90 ± 0.02 measured before inoculation with a pH meter BASIC 20 from Crison Instruments S.A. (Barcelona, Spain) and a water activity meter Novasina Lab Master from Novasina AG (Lachen, Swizerland), respectively. Cuts of slices of "salchichón" with surface of 25 cm^2 and approximately 5 g of weight were aseptically prepared and placed separately in pre-sterilized orthogonal receptacles made of methacrylate, where the humidity was kept by a saturated K_2SO_4 solution (water activity 0.94) placed at the bottom of the receptacles. The samples were inoculated separately on the surface with spores at a concentration of 4 log cfu/cm^2 of: a) the commercial *P. nalgiovense*, b) a well-known OTA-producing *Penicillium verrucosum* Pc4 isolated from meat products (Rodríguez, Rodríguez, Luque, Justesen, & Córdoba, 2011) and c) a mix of the abovementioned strains of *P. nalgiovense* and *P. verrucosum*. In addition, negative controls from non-inoculated slices of "salchichón" were also analysed. Every group of samples (different kinds of inoculation) were ripened and processed for 20 days at 25 °C. Although temperature throughout the ripening process of dry-fermented sausage "salchichón" is usually bellow 15 °C, in some stages of processing may reach higher values (from 18 to 25 °C) as have been previously reported (Lizaso, Chasco, & Beriain, 1999; Martín-Sánchez et al., 2011; Navarro, Nadal, Izquierdo, & Flores, 1997). In addition, in most of the cases at the end of ripening process this product is stored and commercialized at room temperatures where temperatures could reach up to 25 °C. Thus, it is necessary to evaluate the effect of *P. nalgiovense* as protective culture against *P. verrucosum* at those most extreme conditions (abuse temperature) which sausages could be exposed throughout processing or storage conditions.

After incubation time at 25 °C, sampling was carried out by triplicate from each batch. For this, samples of 5 g of dry-fermented sausages were homogenized with 10 mL of Tris–HCl buffer (pH 8.0) in a filter bag BagPage (Interscience, Paris, France) using a Pulsifier equipment. Next, the filtrate which contains spores and the mycelium that grew on the "salchichón" slices was treated for the DNA extraction and for the determination of mould counts (cfu/cm^2). The solid non-filtrate substrate that contains dry-fermented sausage "salchichón" without mycelium and spores was used for OTA extraction and analysis.

2.1.2. Inoculation of commercial dry-fermented "salchichón"

The effect of the inoculation of *P. nalgiovense* was also evaluated in the meat industry in three different commercial processes (Table 1). Thus, these three different dry-fermented "salchichón" types were prepared in the same manufacturing plant belonged to a big company. The three "salchichón" types sampled in this work are widely marketed in Spain. Thus, they are representative of those types usually ripened in dry-fermented sausages manufacturing plants of Spain. In this experiment OTA-producing strains were not inoculated due to the known difficulties derived from the use of toxigenic mould strains in the meat industry. A total of thirty dry-fermented sausages "salchichón" subjected to three different commercial ripening processes (Table 1) were inoculated with *P. nalgiovense*. In each processing, five of them were inoculated by immersion before ripening in a bath with a protective culture of *P. nalgiovense* at a concentration higher than 10^6 cfu/mL. The

Table 1
Types of dry-fermented sausage "salchichón" used in this work and conditions of ripening processes.

Types of dry-fermented sausage "salchichón"	Description of product and processing		
	Diameter	Length	Ripening process
"Casero"	4 cm	16.5 cm	Time: 18 days 3 days: T 5 °C, RH 85% 2 days: T 13 °C, RH 84% 13 days: T 11 °C, RH 84%
"Fuet"	3 cm	27 cm	Time: 21 days 3 days: T 5 °C, RH 85% 18 days: T 12 °C, RH 75%
"Málaga"	3.5 cm	21 cm	Time: 7 days 7 days: T 11 °C, RH 81%