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Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of *Penicillium* isolates

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Abstract

The surface mycoflora of "chorizo de Cantimpalos", a Spanish variety of fermented meat sausage characterised by a natural white covering, has been investigated. Among 54 mould strains isolated, 38 belonged to *Penicillium* subgenus *Penicillium*. The major species found (18 isolates) was identified as *Penicillium commune*, and the other dominant species (13 isolates) was identified as *P. olsonii*. None of the *P. olsonii* isolates produced cyclopiazonic acid, mycophenolic acid, roquefortine C, patulin or ochratoxin A, but all *P. commune* isolates produced cyclopiazonic acid. Toxicity to *Artemia salina* larvae was very high for all *P. commune* isolates investigated, while no isolates of *P. olsonii* studied were toxic to these crustaceans. The results may assist in selection of nontoxic strains, which could be used as surface starters in the manufacture of this type of sausage. The apparent inability to produce penicillin is a valuable characteristic to take into account in the selection process.

Keywords: Surface mycoflora; *Penicillium*; Fermented meat sausage; Mycotoxins

1. Introduction

Moulds play an important role in the manufacture and ripening of cheese and meat products in European countries. A rich variety of such fermented foods is produced in Spain, and in the central region of the country (in Cantimpalos, province of Segovia) a type of fermented meat sausage, "chorizo de Cantimpalos", is popular with consumers. It is characterized by the presence of a white (sometimes green) mould covering which appears naturally on the surface.

The mycoflora of similar fermented meat sausages like salami, made in Italy, has been studied by several authors (Leistner and Eckardt, 1979; Mutti et al., 1992) and some species of *Penicillium* have been found to be responsible for the covering, viz. *Penicillium nalgiovense* and, to a lesser extent, *P. chrysogenum* (Leistner, 1990). This layer of mould is important to the sausage in four ways: (a) it has an antioxidative effect, protecting rancidity from developing and keeping the colour; (b) it allows the development of a favourable microclimate at the surface so that the surface does not become, for example, sticky or slimy; (c) it results in the devel-

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opment of a characteristic flavour, due to the decomposition of fat, proteins and lactic acid; and (d) it gives the sausage its typical appearance (Philipp and Pedersen, 1988). In addition, the moulds may have a protective effect against pathogenic or spoilage microorganisms (Geisen et al., 1992; Leistner, 1994; Singh and Dincho, 1994).

It is generally accepted that the mould strains used in the manufacture of foods should be selected carefully and added during processing in order to assure the safety of the final product. This is particularly important with species in the genus *Penicillium*, many of which are potentially toxigenic (Sweeney and Dobson, 1998). Therefore, from a safety point of view, prior to technological evaluation, isolates must be tested for potential toxigenicity (Leistner, 1986). In addition, several species of *Penicillium* are able to produce penicillin, among which are two species commonly found in meat products, i.e. *P. nalgiovense* and *P. chrysogenum* (Andersen and Frisvad, 1994). Isolates should therefore be screened to make sure that they do not produce this antibiotic. Strain selection also helps in the standardisation of the manufacturing process.

In some countries, e.g. France, the use of mould starters in the manufacture of sausages is common (Leistner, 1986). However, in Spain, this is not usual and even in industrial production, the house ambient mycoflora is the main source of moulds.

The surface mycoflora of Cantimpalos sausage has not previously been investigated. In the study described here, more than 50 strains were isolated from the surface of samples of this variety of fermented meat sausage and identified, and the *Penicillium* isolates investigated for mycotoxin production were assessed for toxicity using a biological test. Finally, the nontoxigenic strains were tested for penicillin production.

2. Materials and methods

2.1. Sampling and isolation of strains

Good quality “chorizos de Cantimpalos”, which had been made without the use of any surface starter, were collected from two major factories in the Cantimpalos area. Three lots of sausages from factory I and two lots from factory II were investigated. From each lot, sausages were collected 5–6 days after the casings had been filled and at the completion of manufacture. In addition, three lots of finished sausages were purchased from the local market.

In the laboratory, mycological samples were taken from the surface of at least two sausages of each lot in two ways: (a) by swabbing the surface of sausage with swabs moistened with sterile 0.1% (v/v) Tween 80 (Panreac, Montflet and Esteban, Barcelona, Spain) and inoculating agar plates by wiping the swabs over the agar, and (b) by directly inoculating plates with small fragments removed from the surface layer using sterile needles. The agar media used were oxytetracycline glucose yeast extract agar (OGYEA, Oxoid, Basingstoke, UK) and dichloran rose bengal chloramphenicol agar, (DRBC, Oxoid). After incubation at 25°C for 5 days, 54 strains of moulds (one strain from each morphological type of colony found on each sample) were isolated from the plates.

2.2. Mould identification

Following the methods of Samson et al. (1995), isolates were identified to genus level on their morphological characteristics. *Penicillium* isolates were identified to species level according to Pitt (1979, 1988). Strains of the main species were sent to three international mycological laboratories for confirmation. In addition, one type A strain was compared with 10 strains of *P. nalgiovense* belonging to six biotypes and one strain of *P. olsonii* by random amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams et al., 1990).

2.3. Mycotoxin production

Production of cyclopiazonic acid was examined in *P. commune* (18 isolates) and of cyclopiazonic acid, mycophenolic acid, patulin, ochratoxin A and roquefortine in 13 isolates of *P. olsonii*. Mycotoxigenicity was investigated by thin layer chromatography (TLC), as is briefly described below.

2.3.1. Mycotoxin extraction

Isolates were grown for 10 days at 25°C on Czapek yeast autolysate agar plates (CYA; Pitt, 1988)

prepared from Czapek–Dox agar (Merck, Darmstadt, Germany) amended with 5 g/l yeast extract (Oxoid). The combined agar and culture were then homogenised in chloroform/methanol (2:1, v/v; 50 ml/plate) and the mixture filtered through anhydrous sodium sulphate. The filtrate was partially evaporated in a rotary evaporator and then to dryness under a nitrogen stream.

2.3.2. Thin layer chromatography

Small aliquots (10–20 μl) of the dried extracts dissolved in 1 ml chloroform were spotted onto silica gel plates (Merck, No. 5554), together with standard solutions of the mycotoxins investigated. The solvents used for each mycotoxin and visualisation methods are specified in Table 1. Detection limits for the individual toxins ranged from 10^{-6} to 10^{-8} g.

2.3.3. Reference standards

Mycophenolic acid was obtained from Fluka (Buchs, Switzerland); roquefortine from the Department of Chemistry, University of Pretoria, South Africa; and cyclopiazonic acid, patulin and ochratoxin A were from Sigma (St. Louis, MO, USA).

2.4. Toxicity to brine shrimp larvae

The toxicity of extracts of cultures on CYA (10 days) of 16 isolates (eight *P. olsonii* and eight *P. commune*, randomly selected) was investigated using the *Artemia salina* mortality test as described by López-Díaz and Flannigan (1997). Larvae were exposed to extracts in a brine shrimp incubator and mortality was determined after 16 and 24 h at 28°C. Previously, it had been verified that there was a high degree of similarity between the mortality of larvae exposed to extracts of CYA culture plates and corresponding plates of yeast extract sucrose agar (YES, Frisvad, 1981). All tests were carried out at least in duplicate.

2.5. Penicillin production

All *P. olsonii* isolates were investigated for penicillin production. Isolates were three-point inoculated on corn steep liquor lactose agar plates (CSLL, Andersen and Frisvad, 1994). After 7 days at 25°C, agar plugs were cut from the colonies and placed on the surface of nutrient agar (Oxoid) plates on which 0.1 ml of a 24-h culture of a penicillin-sensitive strain of *Staphylococcus aureus* (American Type Culture Collection, MD, USA; ATCC 6538P) had been spread (El-Banna et al., 1987b). Plates were incubated at 4°C for 6 h and then at 37°C for 24 h. Any inhibition zone round the agar plugs was considered positive for penicillin production. The experiment was repeated using nutrient agar plates containing penicillinase (Bacto Penase, Difco Laboratories, MI, USA, 1 ml/100 ml medium). *P. chrysogenum* (ATCC 9478) and 1–10 μg of penicillin G (Sigma), applied directly onto the plates, were used as positive controls. Uninoculated plugs of CSLL agar were used as negative controls. All tests were made in duplicate.

Table 1
Systems for detection of mycotoxins by thin layer chromatography

Mycotoxin	Solvent[a]	Visualisation method[b]	Spot colour[c]	Reference
Cyclopiazonic acid[d]	TEF	Erlich solution + HCl vapours, 7 min	Blue	Frisvad et al. (1989)
Mycophenolic acid	TEF	Diethylamine + UV	Blue fl.	Siriwardana and Lafont (1979)
Roquefortine	CM	H_2SO_4 + Q	Blue-grey	Scott and Kennedy (1976)
Patulin	TEF	MBTH + Q	Yellow fl.	Frisvad (1988)
Ochratoxin A	TEF	Anisaldehyde + Q	Turquoise blue fl.	Paterson and Bridge (1994)

[a] TEF, toluene/ethyl acetate/formic acid 90% (5:4:1, v/v/v); CM, chloroform/methanol (2:1, v/v).

[b] Erlich solution, 1% w/v, dimethylbenzaldehyde in 96% ethanol; H_2SO_4, 50% sulphuric acid; Q, heating (105°C/5–10 min); MBTH, methyl benzothiazolinone hydrazone (0.5% aqueous solution); anisaldehyde, *p*-anisaldehyde (0.5% in methanol/acetic acid/concentrated sulphuric acid, 17:2:1, v/v/v).

[c] fl., Fluorescent spots as seen under UV light (365 nm).

[d] Plates previously treated with oxalic acid (8%, w/v) 2 min.

3. Results and discussion

The majority of mould isolates isolated from Cantimpalos sausage were classified in *Penicillium* subgenus *Penicillium* (38/54 isolates). The remaining isolates were in the order *Mucorales*. Owing to their rapid growth on the isolation plates, the presence of these moulds in some samples made the isolation of more slowly growing strains difficult. Mucoraceous strains were present mainly in sausages from factory II and in those obtained at the local market. In these last cases, sausages did not present a typical well developed mould covering, perhaps as a result of post-production contamination with these mucoraceous fungi.

Identification of species in the large genus *Penicillium* is difficult, particularly of isolates belonging to subgenus *Penicillium*, the most frequent types occurring in foods. In our study, on the basis of the macroscopic and microscopic morphology, we could clearly differentiate two main types of *Penicillium*, both belonging to this complex subgenus. These species were initially named type A (13 strains) and type B (18 strains). Another two strains, isolated from two lots of the product from factory I, were classified as *P. chrysogenum*, and five others, from one of the market lots, were not identified to species level.

Both types A and B were consistently isolated from all lots produced in factory I, with type A being isolated most frequently. However, only type B strains were isolated from sausages produced in factory II.

Our morphological study led us to identify species type B as *P. commune*, and the production of cyclopiazonic acid by all type B strains tended to confirm this identification, since most isolates of *P. commune* had previously been found by El-Banna et al. (1987a) to be producers of this toxin. This identification was further confirmed in an authoritative laboratory.

Type A, the other dominant species resembled morphologically *P. nalgiovense*. However, the inability of any of the 13 strains to produce penicillin, a quite consistent product of this species (Andersen and Frisvad, 1994), and the results of DNA analysis, discouraged us from naming the strains as *P. nalgiovense*. The RAPD analysis confirmed that the representative type A could not be allocated to the species *P. nalgiovense*, but demonstrated a similarity to *P. olsonii*. This, along with the identification of one of the reference laboratories as *P. olsonii*, led us to confirm this identification.

In our toxigenicity study, TLC analysis of extracts of colonies grown on CYA demonstrated the production of cyclopiazonic acid by all strains of *P. commune*. None of the five toxins investigated was found in extracts of *P. olsonii* isolates grown on CYA.

Toxicity testing of extracts of *P. commune* and *P. olsonii* isolates against *A. salina* larvae (Table 2) showed the high toxicity of *P. commune* to these crustaceans against the nontoxicity of *P. olsonii* isolates.

The mycoflora of different varieties of fermented meat sausages has been investigated by several authors. Our findings agree with the studies of Dragoni et al. (1991), Kivanc et al. (1992), Roncaglia et al. (1994) and Skinjar and Horvat-Skenderovic (1989) in revealing the dominance of *Penicillium* spp. in such products.

Most studies of fermented meat sausages with a white covering have focused on salami, and again reported the dominance of *Penicillium* in the microflora of the covering. Grazia et al. (1986) found several penicillia in salami, with *P. verrucosum* var. *cyclopium* (syn. *P. aurantiogriseum*, according to Pitt and Hocking, 1997) and, as found in our study, *P. chrysogenum* being the most frequently isolated. Also in salami, Mutti et al. (1992) found that 68% of isolates belonged to this genus. Andersen (1995) found a varied mycoflora on the surface of several

Table 2
Toxicity of extracts of *P. olsonii* and *P. commune* strains isolated from "chorizo de Cantimpalos" to *A. salina* larvae

Strains	Incubation time							
	16 h				24 h			
	NT[a]	ST	T	VT	NT	ST	T	VT
P. olsonii (8)[b]	8[c]	0	0	0	8	0	0	0
P. commune (8)	0	0	8	0	0	0	0	8

[a] Mortality: NT (nontoxic), 0–9%; ST (slightly toxic), 10–49%; T (toxic), 50–89%; VT (very toxic), 90–100%.
[b] Number of strains investigated.
[c] Number of positive strains.

Italian meat sausages, although *Penicillium* predominated once more (96% of isolates). This author isolated both *P. olsonii* (15% of isolates) and *P. commune*, along with *P. chrysogenum* and *P. nalgiovense* (50% of isolates).

During the search for strains which might be suitable as starter cultures of use in production of fermented sausage (Fink-Gremmels et al., 1988; Fink-Gremmels and Leistner, 1990), *P. nalgiovense* was investigated intensively, and some strains are currently used in the manufacture of salami. It is considered to be a nontoxigenic species, although some strains may produce mycotoxins such as cyclopiazonic acid and roquefortine, and a considerable percentage of strains are toxic to *A. salina* larvae (El-Banna et al., 1987a; Fink-Gremmels and Leistner, 1990).

As for other species found on mould-fermented sausage, 88 of 422 *Penicillium* strains isolated by Leistner et al. (1977) produced mycotoxins in broth culture. Leistner and Eckardt (1979) subsequently reported that a high percentage of *Penicillium* strains from Italian and Hungarian salami were toxigenic, and Mutti et al. (1992) found 10 potentially toxigenic *Penicillium* species among 27 isolated from salami, although none was demonstrated as being toxigenic in their study.

P. commune is quite common on foods and is frequent in cheese, its primary habitat, where it is one of the most important causes of spoilage (Pitt and Hocking, 1997). It is believed to be the wild ancestor of *P. camemberti* (Pitt and Hocking, 1997). It produces cyclopiazonic acid, which was produced by all 18 isolates of this species isolated from our samples. Nevertheless, some authors have suggested that this species could be used in the manufacture of cheese (Tzanetakis et al., 1987), and its presence in the chorizo samples taken from both meat factories, investigated in our study, suggests that this species could be of importance in the manufacture of the sausage. Also, it must be considered that other potentially toxigenic species like *P. roqueforti* and *P. camemberti* are used as starter cultures in cheeses. Therefore, the use of *P. commune* strains in the manufacture of chorizo de Cantimpalos must depend, in the first place, on their being unable to produce mycotoxins in the sausage, which has yet to be assessed.

P. olsonii is a species that has been isolated, as well as from sausages (Andersen, 1995), from other foods (Frisvad, 1988; Pitt and Hocking, 1997), and it is considered to be nontoxigenic (Frisvad, 1988; Pitt and Hocking, 1997), as we found in our experiments. The inability of *P. olsonii* to produce penicillin, found in our study, is another property that should be taken into account, since it would favour of the use of this species as starters. Most strains of *P. nalgiovense* isolated from meat products are consistent producers of this antibiotic, which naturally limits their use as starter cultures (Andersen and Frisvad, 1994).

Therefore, according to our study, *P. olsonii* could be considered a new technologically interesting species to be used as a surface culture in the manufacture of chorizo de Cantimpalos.

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