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Mould growth on traditional greek sausages and penicillin production by *Penicillium* isolates

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Abstract

Visible moulds were isolated and identified from traditional Greek sausages from Northern Greece. *Penicillium* species were isolated from 90.8% of visibly mouldy sausages. *Penicillium solitum*, *P. nalgiovense* and *P. commune* species made up 60.6% of the total number of isolates. The most frequently occurring species was *P. solitum* (26.1%). *P. nalgiovense* and *P. olsonii* were found to be positive to penicillin production in an agar assay and further examination for antibiotic production in liquid culture with complex media designed for penicillin production, confirmed their ability for penicillin biosynthesis. Penicillin production by *P. olsonii* is reported for the first time in this study.

Keywords: Mould spoilage; Greek sausage; *Penicillium*; Penicillin biosynthesis

1. Introduction

Traditional sausages are the most commonly produced meat products in Greece. In the past, most Greek rural families prepared them before Christmas from pork meat and fat, chopped and mixed together with salt and seasonings. Seasonings could vary in various parts of Greece, but in general, production technology was the same throughout. The sausage mixture was stuffed in casings prepared from the small intestine of pigs. After production, the sausages were placed in cool rooms with sufficient aeration to allow for drying and consumed within few weeks or in some places over the summer, a period in which substantial weight loss (~30%) occurred and 'fresh' sausages became semi-dried products. Today, although home production still occurs in the traditional way, large quantities are produced throughout the year at butchers' shops and in meat processing companies.

According to Greek Food Legislation (1998), traditional Greek sausages can be produced from lean meat and fat with addition of salt (1.6–2.5%), phosphates, nitrites, monosodium glutamate and ascorbic acid or salt, sugar and various seasonings. Lean or semi-lean pork and beef meat, pork bellies and pork fat can be used as raw materials. The sausage mixture is stuffed in natural cases but the products must be kept chilled until consumption. This product is characterized as fresh and non-cooked and may be partially dried or smoked, fat should not exceed 35%. The product should be cooked before consumption.

The pH in the surface layer of the sausages varies between 4.67 and 6.09, and the a_w value is around 0.96 (Ambosiadis, Soultos, Abrahim, & Bloukas, 2004). The physicochemical, sensory and microbiological qualities of traditional Greek sausages have been characterized in two studies to date (Ambrosiadis et al., 2004; Drosinos et al., 2005). Lactic acid bacteria are the dominant group of microorganisms, while identification of isolates showed

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high counts of *Lactobacillus plantarum* and *Lb. plantarum/pentosus* (Drosinos et al., 2005). Although detailed microbiological profiles of these traditional Greek sausages were presented in both studies, these did not include the surface mycoflora of the products.

Mould growth on traditional Greek sausages can be observed during storage and can be a quality problem. Sausages may become spoiled due to visible mould colonies on the surface and the off-flavors they produce. Mould growth may also represent a health risk because of the possibility of mycotoxin production by several mould species or penicillin production by several species of *Penicillium*. The aim of this study was to describe the spoilage mycoflora of traditional Greek sausages and to identify among *Penicillium* isolates those that produce penicillin.

2. Materials and methods

2.1. Sausage samples

Altogether 96 samples of fresh traditional Greek sausages with visible mould growth were collected from different butchers' shops and food stores from Northern Greece. Analyses were performed within 3 days of collection.

2.2. Isolation of moulds

A small section of the mould colonies representative of each of the different colony types growing on the sausages was plated in 9-cm diameter Petri dishes on malt extract agar (MEA) (Samson, Hoekstra, Frisvad, & Filtenborg, 1995) and incubated for 7 days at 25 °C. Each species isolated from one sausage sample was considered as one isolate. The Petri dishes were inspected and the colonies were sub-cultured onto agar plates according to Samson and van Reenen-Hoekstra (1988), Samson et al. (1995) and Frisvad (1981), as follows: *Penicillium* species were plated onto MEA, Czapek yeast extract agar (CYA), yeast extract sucrose agar (YES), creatine sucrose agar (CREA) and nitrite sucrose agar (NSA). Other genera were plated onto potato dextrose agar (PDA) and MEA. Ready-made media or medium components were purchased from Oxoid (Basingstoke, Hampshire, UK). Agar plates were three point inoculated according to Samson and van Reenen-Hoekstra (1988) and incubated for 7 days, MEA, CYA, YES and PDA at 25 °C and NSA at 20 °C.

2.3. Identification of moulds

Following the methods of Samson et al. (1995), isolates were identified by their morphological characteristics. *Penicillium* isolates were identified at species level according to Pitt (1979, 1988). Fungal morphology was characterized by using a semi-automatic image analysis system consisting of an Olympus microscope (Olympus, New Hyde Park, NY, USA) operated as phase contrast, a CCD camera (Sony, Cambridge, UK), a PC with a frame-grabber, and the image analysis software (SIS, Olympus, Germany). Strains of the main species were sent to an international mycological laboratory for confirmation and identification by molecular analysis and sequences of ITS rDNA. Results were confirmed by morphological observations. 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 (PAPD; Welsh & McClelland, 1990; Williams, Kubelic, Livak, Rafalski, & Tingey, 1990). Also, for identification of *P. commune* a filter paper method and thin layer chromatography (TLC) were used according to Lund (1995a, 1995b).

2.4. Penicillin production

Penicillium isolates were three point inoculated on corn steep liquor lactose agar plates (CSLL) according to Andersen and Frisvad (1994). Following 7 days incubation at 25 °C, agar plugs were cut from colonies and placed onto nutrient agar plates on which 0.1 ml of a 24-h culture of a penicillin-sensitive strain of *Micrococcus flavus* (ATCC 400) had been spread. Plates were incubated at 4 °C for 6 h and then at 30 °C for 24 h. Formation of inhibition zones around the agar plugs indicated penicillin production. Solutions with increasing penicillin G (Sigma Sigma-Aldrich, USA) concentrations were used as controls. The experiment was repeated using nutrient agar plates containing penicillinase (1 ml/100 ml medium) (EC 3.5.2.6, Sigma-Aldrich, USA). *P. chrysogenum* (NRRL 2273) and 1–10 mg of penicillin G applied directly onto the plates, were used as positive controls. Un-inoculated plugs of CSLL agar were used as negative controls. All tests were made in duplicate.

2.5. Determination of penicillin production in liquid culture

Penicillium strains that produced positive results for penicillin production in the above agar assay, were further examined for penicillin production in small-scale liquid fermentations. For each strain, spores obtained from 3 Petri dishes of PDA were inoculated into Erlenmeyer flasks with 100 ml of a chemically defined medium of the following composition, in g l^{-1}: glucose, 40; $NaNO_3$, 3; yeast extract, 2; KCl, 0.5; $MgSO_4 \cdot 7\ H_2O$, 0.5; and $FeSO_4 \cdot 7\ H_2O$, 0.01. The medium pH was adjusted to 6.0 before sterilization. Inoculated flasks were placed in an orbital shaker incubator at 250 rpm and 25 °C for 48 h. 10 ml of each culture was transferred to 100 ml of complex penicillin production medium (CPM) of the following composition, in g l^{-1}: corn steep liquor, 20; lactose, 55; $MgSO_4 \cdot 7\ H_2O$, 3; $CaCO_3$, 10; KH_2PO_4, 7; and 64% potassium phenylacetate, 6.25 ml. The pH was adjusted at 6.8 before sterilization. The cultures were incubated in the orbital shaker incubator at 250 rpm and 25 °C for 120 h. Samples were taken at 1-day intervals and cell-free filtrates were examined for penicillin activity.

3. Results and discussion

The mould species isolated from traditional Greek sausages are shown in Table 1. *Penicillium* species were isolated from 90.8% of the samples of the visibly mouldy sausages. Other genera isolated included *Alternaria*, *Aureobasidium*, *Cladosporium*, *Geotrichum*, *Mucor* and *Phoma*. The most frequently occurring fungal contaminants were *Penicillium solitum*, *P. nalgiovense* and *P. commune*. These species made up 60.6% of the total number of isolates. The most frequently occurring species was *P. solitum* (26.1%).

P. solitum is recognized as a significant pathogen of pomaceous fruit (Frisvad, 1981; Pitt, Spotts, Holmes, & Cruickshank, 1991; Sanderson & Spotts, 1995). This species also causes spoilage of cheese (Hocking & Faedo, 1992; Lund, Filtenborg, & Frisvad, 1995; Kure & Skaar, 2000). *P. solitum* was one of the dominant species (13.02% frequency of isolation) in the contaminant mycoflora of Norvegia and Jarlsberg cheeses according to Kure and Skaar (2000). Isolation of *P. solitum* has been reported from European sausages during manufacturing by Andersen (1995), and from Argentinean salami inoculated with surface culture by Ludemann, Pose, Pollio, and Segura (2004). Surprisingly, *P. solitum* was not among *Penicillium* species isolated from Spanish fermented sausage, chorizo de Cantimpalos by Lopez-Diaz, Santos, Garcia-Lopz, and Otero (2001). According to Pitt and Hocking (1997), limited reports on *P. solitum* isolation reflect lack of recognition and not rarity in foods. Lack of recognition of this species, until recently means that little information exists on its physiology. However, it is a typical member of *Penicillium* subgen, in showing ability to grow at low temperatures and a_w, and absence of growth at 37 °C. *P. solitum* do not produce significant mycotoxins (Frisvad & Filtenborg, 1989) and there are no reports in the literature concerning antibiotic production by this species. It is known however that *P. solitum* produce lipases, compactins and other secondary metabolites (Jong, Birmingham, & Ma, 1994; Surensen, Ostenfeld Larsen, Christofersen, Nielsen, & Anthoni, 1999). The samples in the present study were refrigerated which may explain why *P. solitum* was one of the dominant species.

Next to *P. solitum*, *P. nalgiovense* was isolated most frequently from traditional Greek sausages at 18.8%. *P. nalgiovense* was originally found in cheese, but the species has been isolated from salami-sausages (Samson & van Reenen-Hoekstra, 1988). Today, the main niche occupied by*P. nalgiovense* is as a starter culture for fermented meat products in Europe. Isolation from cheese is rarely reported (Lund et al., 1995). The possibility of mycotoxin production by *P. nalgiovense* has been widely investigated. Most isolates show very low toxicity, and selection of non-toxigenic strains has been successful (Leistner & Pitt, 1977; Fink-Gremmels, El-Banna, & Leistner, 1988; Hwang, Vogel, & Hammes, 1993; Andersen, 1995). Some isolates produce penicillin, reflecting the species derivation from *P. chrysogenum* (Laich, Fierro, Cardoza, & Martin, 1999), while successful attempts have been reported to construct strains with a disrupted *pcb*AB gene, the first gene of the penicillin biosynthetic pathway (Laich, Fierro, & Martin, 2003; Fierro, Laich, Garcia-Ricoo, & Martin, 2004).

P. commune was also isolated from traditional Greek sausages at high frequency (15.7%). This species, which is believed to be the wild type ancestor of *P. camembertii*, is quite common in foods and its primary habitat is cheese (Tzanetakis, Litopoulou-Tzanetaki, & Manolkidis, 1987), of which it is the principal cause of spoilage (Pitt & Hocking, 1997; Kure & Skaar, 2000). *P. commune* has been isolated at a higher frequency compared to other *Penicillium* species from Istrian dried ham at both the pre- and ripening stages by Comi, Orlic, Redzepovic, Urso, and Iacumin (2004). *P. commune* grows rapidly at refrigeration temperatures and is capable of growth below 0.85 a_w. Most isolates of this species produce cyclopiazonic acid (Frisvad & Filtenborg, 1989; Lopez-Diaz et al., 2001), a toxin most often described under *P. camembertii*, and a variety of other possibly toxic compounds (Pitt & Hocking, 1997). However, some authors have suggested that this species can be used in the manufacture of cheese (Tzanetakis et al., 1987) or chorizo de Cantibalos sausages (Lopez-Diaz et al., 2001) despite the fact that all chorizo isolates, in that study were found to be very toxic to larvae of *Artemia salina*. This is not unreasonable since other potentially toxigenic species such as *P. camembertii* and *P. roqueforti* are used as starter cultures. In most reports however, *P. commune* was the major contaminant of cheese and has also been implicated as a cause of the 'phenol defect' in Italian hams during ripening (Spotti, Mutti, & Campanini, 1988). Frequent isolation of *P. commune* from Istrian hams however, was not implicated in any visible defects or toxic metabolite production (Comi et al., 2004).

Table 1
Isolated mould species from visibly mouldy traditional Greek sausages

Species	No. of sausages infected	Frequency of isolation (%)
Alternaria alternata	1	1.0
Aureobasidium pullulans	2	2.1
Cladosporium cladosporioides	1	1.0
Geotrichum candidum	1	1.0
Mucor circinelloides	2	2.1
Mucor racemosus	1	1.0
Penicillium commune	15	15.7
Penicillium echinulatum	5	5.2
Penicillium expansum	4	4.2
Penicillium italicum	4	4.2
Penicillium nalgiovense	18	18.8
Penicillium olsonii	8	8.3
Penicillium oxalicum	6	6.3
Penicillium solitum	25	26.1
Penicillium verrucosum	1	1.0
Penicillium viridicatum	1	1.0
Phoma glomerata	1	1.0
Total	96	100

The frequency of isolation of *P. olsonii* was 8.3%. This species comprised 15% of isolates from a large number of mould-ripened sausages in the study of Andersen (1995). It has also been isolated at high frequency from Spanish fermented sausages by Lopez-Diaz et al. (2001) and all isolates were found to be non-toxic to *Artemia salina* larvae. *P. olsonii* was isolated at low frequency from Argentinean salami by Ludemann et al. (2004) and shown to have proteolytic and lypolytic activities. *P. olsonii* is able to germinate and form microcolonies at 5 °C but does not produce mycotoxins (Pitt & Hocking, 1997).

P. oxalicum frequency of isolation was 6.3%. Isolation of this fungus from fermented sausages has been reported by Andersen (1995). Several of the fungi sporadically isolated are common airborne ones and may be considered as a minor contamination problem.

All isolated *Penicillium* species were tested for penicillin production and were negative except of *P. nalgiovense* and *P. olsonii* (Table 2). *P. nalgiovense* isolates from meat products appear to be very frequent producers of penicillin (Andersen & Frisvad, 1994; Farber & Geisen, 1994; Laich et al., 1999). Significant amounts of penicillin were found *in situ* in the casing and outer layer of salami meat during the early stages of the curing process, coinciding with fungal colonization, but no penicillin was detected in the cured salami in the study of Laich et al. (1999). Biosynthesis of penicillin by *P. olsonii* is reported for the first time in the present study. Examination of samples from shake flask cultures with complex medium revealed maximum production levels of 35 μg/ml for *P. nalgiovense* (at 100 h) and 4 μg/ml for *P. olsonii* (at 120 h). In a recent study by Lopez-Diaz et al. (2001), *P. olsonii* was found to be unable to produce penicillin and the authors suggested that the particular fungus could be considered a new, interesting species to be used as a surface culture in the manufacture of Spanish chorizo de Cantipalos. In our study, *P. olsonii* cultures from all isolated colonies produced penicillin, a fact that contrasts with the findings of Lopez-Diaz et al. (2001). This is not unusual however, since different strains within the same species may exhibit different properties, but further studies to investigate the potential for in situ production of penicillin and also identification of the penicillin gene cluster in this fungus would be interesting.

Table 2
Penicillin production by *Penicillium* isolates from traditional Greek sausages

Species	Positive to penicillin production
Penicillium commune	−
Penicillium echinulatum	−
Penicillium expansum	−
Penicillium italicum	−
Penicillium nalgiovense	+
Penicillium olsonii	+
Penicillium oxalicum	−
Penicillium solitum	−
Penicillium verrucosum	−
Penicillium viridicatum	−

There are no reports in the literature on other properties of interest regarding this fungus, such as protease or lipase production or the production of desirable volatiles.

This study has identified mould species that cause contamination of traditional Greek sausages. Most of the species isolated at high frequencies are capable of producing mycotoxins or penicillin, as demonstrated in liquid culture. Care therefore has to be taken during production and storage. In previous publications on traditional Greek sausages the contaminant surface mycoflora was not assessed. This study thus completes the microbiological profile of a very popular type of European sausage.

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