*International Journal of Food Microbiology*, 13 (1991) 249–256



FOOD 00412

# Mycoflora and toxigenic *Aspergillus flavus* in Spanish dry-cured ham

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(Received 17 July 1990; revision received 20 February 1991; accepted 28 March 1991)

Sixty-five dry-salted hams were analysed. *Aspergillus* and *Penicillium* were the dominant genera. In general, the mould flora was dominated by *Aspergillus* spp. and primarily *A. glaucus*, *A. fumigatus*, *A. niger* and *A. flavus*. *A flavus* was found in 16 hams and 9 out of 16 strains examined produced aflatoxins 'in vitro'. Surface samples of dry-salted hams showed growth of inoculated *A. parasiticus* NRRL-2999 strains and production of aflatoxins in low levels at 25 and 30 °C. It is concluded that the presence of toxigenic strains in Spanish dry-salted ham does not constitute a health risk.

Key words: Dry-cured ham; Mould; *Aspergillus*; *Penicillium*; *Aspergillus flavus*; Aflatoxin

## Introduction

Country-cured hams are dry-cured meats commonly produced in Spain. Spanish ham in its most interesting and aromatic varieties is cured for certain periods of time during which it develops a superficial mould growth which is characteristic for each area of production. The presence of moulds on the surface is primarily associated with long periods of maturation. The fungal flora is fundamental, together with the lipid composition, for the final flavour of the ham. However, it should be realized that many of the moulds present in ham not only produce desired changes; but may also synthesize mycotoxins which could cause serious health risks for the consumer.

The importance of moulds in the maturing of the ham and the possible presence of toxigenic moulds determined our objectives of this work, i.e. to analyse the final mycoflora of cured Spanish ham and investigate the toxigenic potential of isolated strains of *A. flavus*.

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## Materials and Methods

*Samples of ham*

The technological characteristics of the curing process of Spanish ham are generally those specified by Carrascosa (1989) which briefly can be described as follows: dry-salting is carried out for 8–10 days at 5 °C and the hams are then brushed to eliminate the excess salt. During this phase, water activity ($a_w$) is reduced to 0.95. The ham is left for 40–45 days at 5 °C. At the end of this stage the $a_w$ is approximately 0.84. This is followed by a drying period of 80–100 days which is divided into four stages: (i) 25–30 days at 14 °C; (ii) 20–25 days at 25 °C; (iii) 25–30 days at 32 °C; and (iv) 10–15 days at 35 °C. The $a_w$ at the end of this process is 0.91. Maturation takes place when stored at room temperature (dry Continental climate) varying according to the season and lasting between 1 and 18 months. The $a_w$ is about 0.90 at the end of maturation. The surface NaCl concentration of the ham is about 16% (w/w) in the finished product, which significantly influences the composition of the fungal flora and its metabolic activity.

*Sampling*

Sixty-five surface samples of commercially produced hams were analysed. Samples were obtained by scraping the surface of the hams (approx. 10 $cm^2$) with a sterile lancet.

*Enumeration and identification of moulds*

After dispersion and homogenization in sterile peptone (0.1% w/v) for 5 min using an MSE Homogenizer (Vernitron Medical Products, Multimix Lourdes, Mod. MM-1B). Moulds were enumerated using duplicate surface spread plates (Flannigan, 1973). Media used for moulds were potato dextrose agar (PDA) (Difco). PDA plates were incubated at 25 °C for 5 days and at 37 °C for 3–5 days, respectively.

Fungal colonies were identified according to Smith (1963), Raper and Fennell (1965), Ainsworth et al. (1973), Alarca Salat (1980), Dragoni et al. (1980) and Fassatiova (1986).

*Screening for of aflatoxin-producing strains of Aspergillus flavus*

Identification of isolates as *A. flavus*, in the sense of the series consisting of *A. flavus* Link and *A. parasiticus* Speare (Thom and Raper, 1945), was based on colony colour and gross morphology of conidial heads (Raper and Fennell, 1965). A range of such isolates, selected on the basis of frequency of occurrence in hams, were tested for production of orange pigment on *A. flavus* and *A. parasiticus* agar (AFPA) (Pitt et al., 1983). Production of aflatoxin was detected using aflatoxin production agar (APA) (Hara et al., 1974). As APA appeared to be inhibitory to the germination of spores of some isolates (data not included), all APA plates were inoculated with 4-mm plugs cut from the margins of 4-day PDA plate cultures, and

examined for fluorescence under UV light (360 nm) after incubation at 25 °C for 12 days.

*Extraction and detection of aflatoxins by thin-layer chromatography from fluorescent medium*

The procedure of Hara et al. (1974) was followed except that 30 g agar medium from duplicate fluorescent plates were macerated in 50 ml distilled water and extracted in 50 ml chloroform (parity 99%; Panreac). The mixture was centrifuged at $3000 \times g$ for 10 min, the chloroformic phase was poured off and the agar, together with the aqueous phase, was again collected in a Waring Blender and extracted once more with 25 ml of chloroform. The chloroform extracts were combined and concentrated to dryness at 55 °C in an atmosphere of nitrogen.

Thin-layer chromatography was carried out as described by Scott et al. (1970). The quantification of aflatoxins was carried out by the fluorimetric method (Stacchini and Manzone, 1965). A Perkin-Elmer model 3.000 spectrofluorometer was used. The primary filter used was 365 nm, the secondary filter for $B_1$ and $B_2$ was 430 nm, and for $G_1$ and $G_2$ it was 450 nm. The detection limits of the technique were 4–5 $\mu$g/kg for aflatoxins.

*Production and detection of aflatoxins in ham*

*A parasiticus* NRRL-2999 was tested for its ability to produce aflatoxins during growth on surface samples of fully matured ham containing approx. 16% (w/w) NaCl.

The sample of ham (100 g), contained in a 250-ml conical flask, was previously flame-sterilized on the surface. Duplicate flasks were inoculated with 1 ml of a spore suspension prepared by adding 10 ml of sterile 0.01% Tween 80 to a 4-day PDA plate culture and detaching spores by gentle scraping with a sterile loop. Flasks were incubated at different temperatures: 5, 15, 25 and 30 °C for 20 days and the ham with heavy mould growth was then macerated for 5 min in 200 ml acetonitrile-water (90:10) using a Waring Blender and further extracted and purified extracted and purified as described by Obioha et al. (1983).

For fluorimetric estimation of aflatoxins known volumes of chloroform, extracts were applied as a series of discrete spots along the base line of silica gel plates, which were developed first in petroleum ether to remove extraneous fluorescent material, dried and then developed in chloroform:acetone (180:20) (Gimeno, 1979). Quantification of aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$ was carried out by the fluorimetric method (Stacchini and Manzone, 1965), as described above.

## Results and Discussion

The most widely represented genera was *Aspergillus* and *Penicillium*, followed by *Pullularia, Paecilomyces, Monilia, Cladosporium, Alternaria* and other genera (data not shown). The first two genera were isolated from about 50% of the samples. Both *Aspergillus* and *Penicillium* have been recovered in high numbers in

TABLE I

*Aspergillus* isolates from surface of hams

| Species | Positive samples | % of total samples |
|---|---|---|
| *A. glaucus* (group) | 35 | 53.9 |
| *A. fumigatus* (group) | 18 | 27.7 |
| *A niger* (group) | 17 | 26.2 |
| *A. flavus* (group) | 16 | 24.6 |
| *A. ochraceus* | 3 | 4.6 |
| *A. flavipes* | 3 | 4.6 |
| *A. restrictus* (group) | 2 | 3.1 |
| *A. nidulans* | 1 | 1.5 |
| *A. terreus* | 1 | 1.5 |
| *A. versicolor* | 1 | 1.5 |
| *A. wentii* | 1 | 1.5 |

similar studies (Leistner and Ayres, 1968; Bullerman et al., 1969; Hadlock et al., 1976; Cantoni et al., 1977; Dragoni and Cantoni, 1979). Cantoni et al. (1977) and Huerta et al. (1987) found that *Aspergillus* was dominant in cured hams, followed by *Penicillium*. The incidence of *Pullularia*, *Paecilomyces*, *Monilia*, *Cladosporium*, and *Alternaria* was always lower than that of the two previously mentioned genera as reported by the above-named authors.

Among the *Aspergillus* spp. those belonging to the *Glaucus* group were detected most frequently, followed by *A. fumigatus*, *A. niger* and *A. flavus* (Table I). In general, the increase in temperature with drying time and maturation at room temperature could be a favourable condition for *Aspergillus* in the later stages of the process.

*A. glaucus* are recorded as being more xerophilic than *A. niger*, *A. ochraceus* and *A. fumigatus*. Leistner and Ayres (1968) isolated mainly *A. ruber* in American country-cured hams at the final stages of curing, together with lower numbers of other groups. Among these, the *A. restrictus* was predominant. Since most species included in the *A. glaucus* group are strongly xerophilic, the dominance of these species in our examinations of Spanish dry-cured hams could be explained by the lower final $a_w$ value (0.90) of this product when compared to the majority of cured Italian and American hams, having $a_w$ of between 0.92 and 0.94 (Huerta et al., 1987).

Out of the aspergilli isolated from 65 Spanish country cured hams, only 16 were *A. flavus*. All of the 16 strains of *A. flavus* isolated produced orange pigment on AFPA, confirming the identification based on gross morphology, but only nine strains (56.2%) produced fluorescent zones on APA. Thin-layer chromatography of chloroform extracts of fluorescent agar showed that five out of nine strains tested produced detectable amounts of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$. One strain produced only aflatoxins $B_1$, $B_2$ and $G_1$. Two strains produced aflatoxins $B_1$ and $B_2$, and one only aflatoxin $B_1$ (Fig. 1). Other investigations (Leistner and Ayres, 1968;

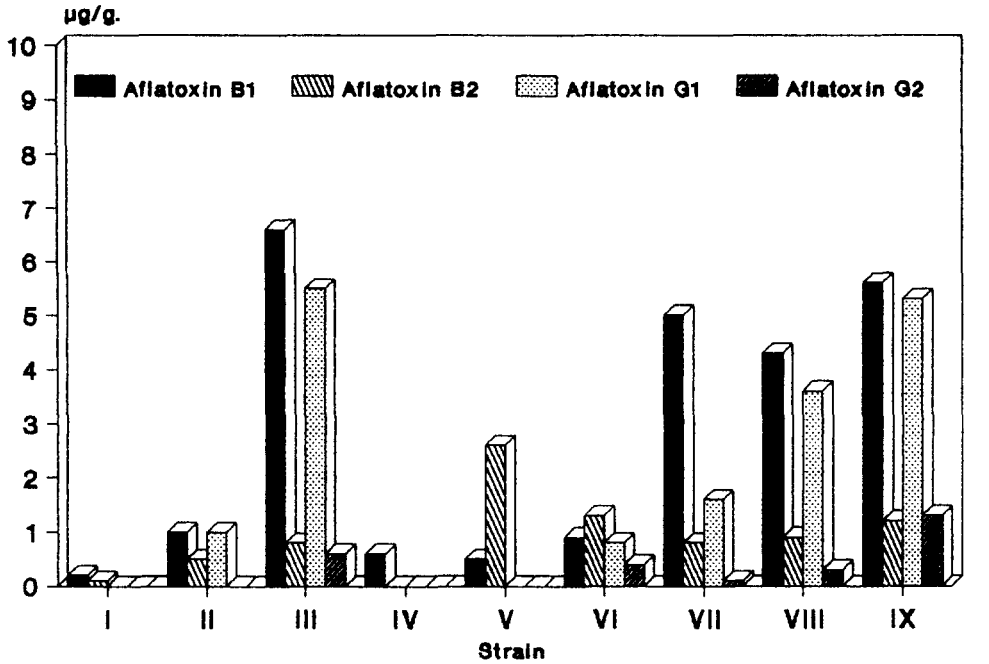


Fig. 1. Production of aflatoxins in APA medium (Hara et al., 1974) by nine strains of *Aspergillus flavus* isolated from ham.

Strzelecki et al., 1969) indicated that the percentage of toxigenic strains of *A. flavus* isolated from cured hams varied between 66.6 and 90%. With regard to the amount of aflatoxins, Strzelecki et al. (1969) reported a synthesis of group B aflatoxins of between 0.98 and 1.7 µg/ml in laboratory medium, by *A. flavus* strains isolated from cured hams and with no group G aflatoxins detectable. These results are slightly lower than those obtained by us (Fig. 1).

The presence of toxigenic *A. flavus* in cured hams raises the question whether hams can be a source of aflatoxins (Huerta et al., 1987). We therefore carried out an experiment whereby the surface of the ham was inoculated with a toxigenic strain of *A. parasiticus* NRRL-2999. The results are shown in Table II. Growth

TABLE II

Production of aflatoxins on ham inoculated with *A. parasiticus* NRRL-2999

| Temperatures | Aflatoxins (µg/kg) | | | | |
|---|---|---|---|---|---|
| | $B_1$ | $B_2$ | $G_1$ | $G_2$ | Total |
| 5 °C | – | – | – | – | – |
| 15 °C | – | – | – | – | – |
| 25 °C | 0.42 | – | 0.30 | – | 0.72 |
| 30 °C | 1.37 | – | 0.36 | – | 1.73 |

was not recorded at 5 °C. At 15 °C visible growth was seen, but aflatoxin synthesis did not occur. The maximum amount of aflatoxins synthesized was as low as 1.73 μg/kg at 30 °C and 0.72 μg/kg at 25 °C. Leistner and Ayres (1968) found between 0 and 90 μg/kg in cured hams kept at 25 °C.

In general, we have noted conflicting information on the synthesis of mycotoxins in cured meats (Bullerman et al., 1969; Ciegler et al., 1972; Alperden et al., 1973; Wu et al., 1974). Although toxigenic strains occur on ham, it remains to be demonstrated that aflatoxins are actually produced in the Spanish dry-cured ham and based on the present examinations we do not consider the presence of toxigenic strains of *A. flavus* to be a health risk for the consumer.

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