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International Journal of Food Microbiology 96 (2004) 29–34

INTERNATIONAL JOURNAL OF Food Microbiology

www.elsevier.com/locate/ijfoodmicro

Moulds isolated from Istrian dried ham at the pre-ripening and ripening level

Giuseppe Comi[a,*], Sandi Orlic[b], Sulejman Redzepovic[b], Rosalinda Urso[a], Lucilla Iacumin[a]

[a] *Dipartimento di Scienze degli Alimenti, Facoltà di Agraria, Università degli Studi di Udine, Via Marangoni, 97, 33100 Udine, Italy*
[b] *Department of Microbiology, Faculty of Agriculture, University of Zagreb, Svetosimunska, 25 Zagreb, Croatia*

Received 3 October 2003; received in revised form 1 March 2004; accepted 22 March 2004

Abstract

The aim of this study is to define the mould strains growing on the surface during the pre-ripening and the ripening phases of Istrian ham, and their toxic potential. The mould microflora was predominantly represented by five genera, which were isolated on the ham surfaces of three different producers investigated. The identified species were similar in the both tested periods, demonstrating that the contamination came mainly from the air and the ripening chambers (seasoning rooms), rather than the raw meat. *Eurotium* spp., *Aspergillus* spp. and *Penicillium* spp. were the main strains isolated. The presence and growth of the different strains depended on the temperature of ripening and the relative humidity in the ripening chambers, since the hams were home made products and not matured in controlled conditions. The toxic potential of isolated strains was also investigated. None of the tested moulds can produce mycotoxins and for this reason the Istrian hams do not represent a health hazard.

Keywords: Moulds; Dried ham; Mycotoxins

1. Introduction

Istria is one of the most remarkable regions for dried ham production in Croatia. This peninsula is located in the north region of Adriatic Sea. The climate is predominantly Mediterranean, with temperate winter and hot summer. Winter is characterised by cold northeastern wind called Bura. The production practice of dried ham dates back to the 1600s.

Istrian dried ham is a specific product that is different from all the other dried hams (Parma, San Daniele, Spain, Dalmatia ecc.). The most suitable period for starting the Istrian dried ham production is from November to February, due to good climatic conditions. It is made from heavy pig (150–220 kg) and the production process has four phases: salting (around 2 weeks), pressing (around 7 days), drying (around 3 months) and ripening (8–14 months). At the beginning of salting and drying stage pepper, garlic and laurel are added. The whole production process is very long (12–18 months). During this period, on the surface of dried ham different moulds are formed. Several biochem-

* Corresponding author. Tel.: +39-4-32-590745; fax: +39-4-32-590719.
E-mail address: giuseppe.comi@dsa.uniud.it (G. Comi).

0168-1605/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2004.03.005

ical changes are observed, including degradation of fat and proteins by hydrolysis and the development of the characteristic flavour of these meat products. The main characteristic of Istrian dried ham is that it is produced without pig skin. The reason is only tradition, because of the high price of pig fat in the past.

The moulds on the surface of the dry-cured ham have been studied in great details (Leistner and Ayres, 1968; Bullerman et al., 1969; Sutic et al., 1972; Hadlock et al., 1976; Dragoni and Cantoni, 1979; Dragoni et al., 1980a,b; Montel et al., 1986; Huerta et al., 1987; Rojas et al., 1991; Spotti et al., 1988, 1989, 2001a,b; El-Kady et al., 1994; Nunez et al., 1996; Sosa et al., 2002). The *Penicillium* spp., *Aspergillus* spp. and *Eurotium* spp. seem to be the most present during all stages of the ham production. The succession or the prevalence of one or other genus on the surface of the industrial hams depends on the temperature of ripening on the moisture of the meat and on the relative humidity, used in climatized (controlled) ripening chambers. Vice versa in the home-made hams the presence of the different moulds is subject of the climate conditions in the chambers used for production. In some cases, the mould growth is so high that there is a real problem with the presence of toxigenic moulds (Nunez et al., 1996). To prevent the growth of undesirable moulds, it is suggested to use the non-toxigenic strains, as starters. In this case, the health hazard due to the mycotoxins presence can be minimized.

In many European countries (Italy, Spain, Germany, Croatia), dry sausages with a mould coating are very popular, but dried ham coated with moulds is specific for Istria. The surface mould contributes to external appearance of the product and provides a ''microclimate'' during the ripening. The usage of controlled strains can avoid the development of toxinogenic moulds, which can grow during natural fermentation of ham. The moulds present on the surface could contribute to the taste, odour and storage quality.

To our knowledge, the mould content on Istrian dried ham has not yet been reported. The aim of this study is to define the mould strains growing on the surface during the pre-ripening and the ripening phase of Istrian ham and their toxic potential.

2. Materials and methods

2.1. Samples and moulds identification

Eighty-one hams were analysed during the pre-ripening (2 months after salting) and end-ripening (15 months after salting) phase from three ham producers (27 hams per producers) in the Istrian area. From the surface of each ham, a portion of aerial mycelium was taken by sterile platinum inoculating loop. The samples were collected in three different parts of the seasoning rooms: front, middle and back.

The samples were streaked onto Malt Agar (Malt extract 20 g, yeast extract 5 g, agar 20 g, distilled water 1 l, pH 6.0), which were incubated at 30 °C for 3–5 days. Then, the colonies of the moulds were inoculated in three different agars: Czapek Dox Agar (Pitt, 1973), Malt Agar (Malt extract 20 g, yeast extract 5 g, agar 20 g, distilled water 1 l, pH 6.0) and Salt-Malt agar (Malt extract 50 g, NaCl 50 g, agar 20 g, distilled water 1 l, pH 6.2) and identified according to Raper and Fennell (1965), Ainsworth et al. (1973) and Samson et al. (2000).

2.2. Mycotoxin production in "vitro"

Different methods were used for determination of the mycotoxin production in 60 strains isolated from dry ham.

Method (a): All isolates were cultivated on potato-dextrose (Oxoid, Italy) and 6.5% NaCl (common level in dry ham) liquid medium for 10 days. The mycotoxins were extracted according to El-Kady et al. (1994);

Method (b): All isolates, potential Aflatoxin producers, were inoculated at the center of solidified agar medium in glass petri dishes and incubated at 28 °C in the dark for 10 days. The medium, the observation of the fluorescence and the mycotoxin extraction were according to Hara et al. (1974);

Method (c): All isolates were inoculated in the center of Malt extract peptone agar (20% meat extract, 0.25% peptone, 2% agar and 6.5% NaCl) and incubated at 28 °C for 10 days. The mycotoxin extraction was done according to Sosa et al. (2002). Each test and each extraction from each medium was done in triplicate.

2.3. Mycotoxin detection

Diffchamb (Italy) tests were used in order to detect the different mycotoxins. Ridascreen tests for Aflatoxin B1, B2, G1 and G2, Ochratoxin A, Deoxynivalenol, Fumonisin and Zearalenone were used according to the manufacturer.

3. Results and discussion

A total of 382 strains were isolated. From the analysed hams during the pre-ripening phase were isolated 194 strains (161 true and 33 sterile mycelium) and 188 strains from end-ripening phase (146 true and 42 sterile mycelium). All the isolated strains belonged to 17 species (Tables 1 and 2). The mycoflora is mainly represented by five genera, in all the three different producers investigated. The identified species were similar in both tested periods, demonstrating that the contamination came mainly from the air and the seasoning chambers, rather than the raw meat. On the surface of the investigated hams, a different number of moulds strains was observed, considering the zone of taken samples and the phase of ripening.

Table 1
Number of moulds isolated in Istrian ham at the pre-ripening phase

Mould species	Pu			Sa			Zm		
	A	B	C	A	B	C	A	B	C
Penicillium frequentans	3	0	4	2	0	0	3	2	2
Penicillium verrocosum	4	0	0	0	2	0	0	4	0
Penicillium lanoso-coeruleum	1	2	2	1	2	1	1	3	0
Penicillium lanoso-griseum	1	3	0	1	4	0	2	3	0
Penicillium citrinum	0	0	0	0	2	0	0	0	0
Penicillium chrysogenum	2	2	0	0	3	0	0	3	0
Penicillium commune	2	2	0	0	2	0	1	0	0
Penicillium expansum	1	2	0	0	0	0	0	3	1
Aspergillus flavus	0	1	0	0	3	0	0	2	0
Aspergillus repens	1	2	0	1	0	0	1	3	0
Aspergillus parasiticus	1	1	0	2	2	0	1	0	0
Aspergillus candidus	1	1	0	0	0	0	0	0	0
Aspergillus ochraceus	1	2	0	0	2	0	1	3	0
Fusarium graminearum	1	2	0	0	2	0	0	1	0
Fusarium culmorum	1	0	0	0	2	0	0	2	0
Mucor racemosus	1	1	0	2	1	2	2	0	0
Eurotium repens	6	2	3	4	3	3	3	3	3
Total isolates	27	23	9	13	30	6	15	32	6

Pu, Sa, Zm: different manufactures;
A—front, B—center and C—back of seasoning room.

Table 2
Number of moulds isolated in Istrian hams at the end-ripening phase

Mould species	Pu			Sa			Zm		
	A	B	C	A	B	C	A	B	C
Penicillium frequentans	1	0	3	1	0	0	1	1	1
Penicillium verrocosum	2	0	0	0	1	0	0	3	1
Penicillium lanoso-coeruleum	0	2	2	0	2	0	2	3	0
Penicillium lanoso-griseum	2	2	0	1	5	0	1	4	0
Penicillium citrinum	0	0	0	0	0	0	0	0	0
Penicillium chrysogenum	1	2	0	1	1	0	0	2	0
Penicillium commune	3	2	1	0	4	0	0	0	0
Penicillium expansum	1	1	0	0	0	0	0	2	1
Aspergillus flavus	0	0	0	0	2	0	0	1	1
Aspergillus repens	2	3	1	2	3	0	2	3	0
Aspergillus parasiticus	0	1	0	1	1	0	1	0	0
Aspergillus candidus	0	0	0	0	0	0	0	0	0
Aspergillus ochraceus	1	1	1	1	1	0	1	2	0
Fusarium graminearum	1	1	0	0	1	0	0	0	0
Fusarium culmorum	0	0	0	0	3	0	0	3	0
Mucor racemosus	0	0	0	1	0	1	0	0	0
Eurotium repens	5	5	4	3	7	4	2	5	5
Total isolates	19	20	12	11	31	5	10	29	9

Pu, Sa, Zm: different manufacturers;
A—front, B—center and C—back of seasoning room.

Difference in the number of the isolated strains in the three zones was also noted between the three ham producers investigated. The higher percentage of the isolates found in the centre zone is probably due to the stagnant humidity in this zone, which was always more than 70% in all ham producers. Vice versa, in the front or in the back zone, the presence of the doors and the windows allowed a circulation of the air and change in the humidity. In both zones, the relative humidity varied in all ham producers from 5% to 80%.

Penicillium, *Aspergillus* and *Eurotium* species represented more than 97% of the isolates. Both *Aspergillus* and *Penicillium* have been recovered in high number in similar studies (Leistner and Ayres, 1968; Bullerman et al., 1969; Hadlock et al., 1976; Dragoni and Cantoni, 1979; Dragoni et al., 1980a,b; Rojas et al., 1991; Spotti et al., 1989; Nunez et al., 1996). *Eurotium* species were the dominant strains isolated in all three producers, either in pre or in ripening phase. The succession of *Penicillium* spp. by *Aspergillus* spp. and *Eurotium* spp was not noticed, as reported by various authors (Leistner and Ayres, 1968; Spotti et al., 1989; Hernàndez and Huerta, 1993) in hams with low Aw or ripened under low relative humidity. In this

study, the frequency of *Eurotium* strains was high from the early stages of pre-ripening and its percentage increased during the post-drying and till the end-ripening phase. Probably, the increase is related to the high resistance of its spores to drying of the air and the ham. *Eurotium* spp. are selected in high frequency at the different degrees of Aw of the hams, which after pre-ripening is less than 0.92, and the relative humidity (RH) was equal or less than 80% for great part of the pre- or end-ripening period. *Eurotium repens*, the only *Eurotium* species found in this study, can grow in dry areas (RH < 80%). It is found on the surface of different dried meat products, also on the ham, and can rapidly become the main strain. Rodriguez et al. (1998) and Nunez et al. (1996) found the prevalence of *Aspergillus* and *Eurotium*, when the surface Aw values of hams reached 0.88–0.79. Previously, Spotti et al. (1989) had found that *E. repens* could represent the 66% of the total flora on dry-cured hams and that its growth could increase from pre- to end-ripening phase. This species can positively influence the ripening of the hams (Leistner, 1984; Huerta et al., 1987). It seems that the typical aroma of hams can also be due to the growth of this strains on the muscle portion and the presence on the ham surface is a good indicator for ripening evaluation and reaching the reduced value of Aw (Leistner, 1984).

Pennicillium and *Aspergillus* are represented by different species. These genera did not dominate. Data are not similar with the ones of Cantoni et al. (1977) and Huerta et al. (1987). Both authors found that *Aspergillus* was dominant in dry-cured hams and they considered the presence of *Penicillium* as occasionally contamination.

Penicillium frequentans, *P. verrucosum*, *P. lanoso-coeruleum*, *P. lanoso-griseum*, *P. chrysogenum*, *P. commune* and *P. expansum* were the most frequently isolated *Penicillium* strains in both pre-ripening and end-ripening time. *P. citrinum* was only occasionally found. All the species are xerophilic. In particular, *Penicillium verrucosum*, *P. chrysogenum* and *P. citrinum* can grow and predominate in food with low Aw and RH (Pitt and Hocking, 1985; Grazia et al., 1986). However, on meat products, they can also grow well in high moisture or RH. In this study, *Penicillium* spp. were most frequently found on the surface of the hams kept in the middle zones, where the RH was for the most ripening time, higher than 80–85%. Data were quite similar in all three ham producers. *Penicillium* spp. never predominated over the *Eurotium* spp., not in the early stage of ham production nor in the pre or ripening. That is in contrast with the data of Nunez et al. (1996), which found a prevalence of *Penicillium* strains at level of post-salting and end-drying, when the Aw of the ham surface was up to 0.90%. The reason for the low level of the *Penicillium* spp. concentration could be due to the production area (seasoning chamber), which did not had controlled conditions, the temperature and the relative humidity. So the RH and temperature values depended on the external climate conditions, and it was possible that the wind (Bura) being more present in Istria area can dehydrate the surface of the hams in the early stage of their production, favouring the *Eurotium* spp. growth. In addition, Spotti et al. (1989) found that *P. verrucosum* could dominate the mould flora of dry ham only when they were ripened in high relative humidities.

In any case, the growth of some *Penicillium* spp. is not favourable, because they can produce off-flavour and toxic metabolites (Northolt and Bullerman, 1982; Spotti et al., 1988). *Penicillium* spp. and *Penicillium commune*, in particular, are recognized to be responsible for the phenic acid defect. Spotti et al. (1988) demonstrated that hams spoiled by *P. commune* showed a phenol-like off-odour. The odour was localized at the aitchbones of the hams and, for preventing it, they suggested the control of *P. commune* concentration (less than 100 spores), the control of the salt content and the limiting of the RH (lower than 85%) during the ripening. In this study, no off-odours were observed. In particular, no phenic acid defect was found, considering that the frequency of *P. commune* was low.

Aspergillus flavus, *A. repens*, *A. parasiticus*, *A. candidus* and *A. ochraceus* were also found of the surface of the tested hams. They were present in the front and the middle zones of seasoning chambers, in both pre-ripening and ripening phase. They were never found in the back ripening zone. Their frequencies were low and they were only occasionally isolated. This data is not in agreement with the Cantoni et al. (1977), Dragoni et al. (1980a,b), Huerta et al. (1987) and Rojas et al. (1991), which considered *Aspergillus* spp. the dominant mould flora of the cured hams. Rojas et al. (1991) more frequently detected *A. fumigatus*, *A. niger* and *A. flavus*, and demonstrated that the increase in temperature, with

drying time and maturation at room temperature, could be a favourable condition for *Aspergillus* growth, in the later stages of the process.

Fusarium graminearum, *F. culmorum* and *Mucor racemosus* were also present on the surface of the investigated hams. They were only occasionally isolated, either in the three zones of seasoning chambers, or in the three different producers investigated. This species are widespread in nature and in the soil, so they could have contaminated the surface of the hams by the air.

Sixty of the mould strains isolated from the Istrian hams, including *Aspergillus* spp., *Penicillium* spp., *E. repens*, *Fusarium* spp. and *M. racemosus*, were tested in order to investigate their toxic potential. All the strains tested did not produce toxic metabolites in "vitro", despite the use of three different techniques of toxin production. Data are not in agreement with those of Hara et al. (1974), Rojas et al. (1991), El-Kady et al. (1994), Nunez et al. (1996), Spotti et al. (2001a,b), Diaz et al. (2002) and Sosa et al. (2002), which tested the toxic potential of moulds isolated from hams or other meat products. Leistner and Ayres (1968) and Strzelecki et al. (1972) indicated that the percentage of toxigenic strains of *A. flavus* from cured hams varied between 66.6% and 90%. The ranges of Aflatoxin concentrations produced by different isolated mould strains were variable and were 0.98 and 1.7 μg (group B)/ml (Strzelecki et al., 1972), 0.9 and 240 μg/ml (El-Kady et al., 1994), 0.30 and 1.37 μg/kg (Rojas et al., 1991).

In our study, the tested strains could not produce Aflatoxin B1, B2, G1 and G2, Ochratoxin A, Deoxynivalenol, Fumonisin and Zearalenone, despite the species considered. It was impossible to explain the reasons for the lack of toxic metabolite production in the isolated strains. Maybe the reasons can be found in the specific environmental conditions of production area and in the meat of local origin, which can all contribute to the selection of non-toxigenic mould strains.

4. Conclusions

Istrian ham is one of the best products of Croatia. During the pre-ripening and the ripening phase, different moulds are formed on the dried ham surface. The mould flora was variable and is mainly represented by *Aspergillus* spp., *Penicillium* spp. and *Eurotium* spp. *Eurotium* spp. predominate in the early stage of ham production, because of the relative humidity of the environment which is subjected to the external climatic conditions. During the period of production, the climate is characterised by cold northeastern wind called Bura, which rapidly dries the ham surface, favouring the growth of xerophilic moulds such as *Eurotium* spp. All the tested mould strains could not synthesise mycotoxin.

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