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Penicillium Populations in Dry-Cured Ham Manufacturing Plants

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ABSTRACT

Seven ham manufacturing plants were sampled for 1 year to assess the mycoflora present in the air and on hams, with special attention given to potential mycotoxin producers. Temperature and relative humidity were recorded in the ripening rooms. Maturing rooms held hams from 2 to 3 through 6 to 7 ripening months, and aging rooms held hams for the following 6 to 7 months, until the 14-month ripening point, when they were ready for the market. Mean temperatures and relative humidities registered during the study were 14.9°C and 62.4%, respectively, in maturing rooms and 16.3°C and 57.6% in aging rooms. Aspergilli and penicillia, potential mycotoxin producers, were isolated in all the plants from the air and the ham. Aspergilli represented 5% of the isolates, while penicillia were largely dominant, with *Penicillium nalgiovense* being the most represented species (around 60% of the penicillia), followed by *Penicillium nordicum,* with 10 and 26% of the penicillia isolated, respectively, from the air or the ham. Ochratoxin A production ability, checked in vitro at 25°C, was observed in 50% of the *P. nordicum* isolates obtained both from the air and the ham. Air and ham surface contamination by penicillia was greater in the ripening rooms, where higher temperatures were registered. A certain correlation was also observed between air and ham surface contamination. On the basis of this study, *P. nordicum,* the ochratoxin A producer that is notable on proteinaceous substrates, is normally present in ham manufacturing plants in Italy, even though not a dominant species. Further studies are necessary to clarify and ensure if dry-curing conditions minimize the potential risk of ochratoxin A formation in the product.

Dry-cured hams are prepared from the thighs of selected pigs and stored in plants placed in favorable geographic areas for ripening. Basic technological treatments applied to pork thighs are as follows: (i) ham salting and resting (2 to 4 months) at low temperatures (0 to 4°C) and high (75 to 90%) relative humidity (RH); (ii) drying and maturing (3 to 4 months) at mild temperatures (12 to 18°C) and 50 to 70% RH; (iii) application of ''fat'' (a spreadable mince made up of pork fat, rice flour, and spices) on the exposed muscle surface to prevent crusting of the outer muscle layer; and (iv) mid- to long-term aging (8 to 10 months) at temperatures in the range of 15 to 20°C and 40 to 70% RH. Drying, maturing, and aging phases were carried out in separate rooms.

Few fungal species have been isolated from dry-cured hams during the cold salting phase *(24),* while abundant yeast and mold growth is often observed on exposed muscle surfaces during ripening. Presumably, the composition and water activity of hams *(22)* and the temperature and RH of ripening rooms *(12, 24, 25)* are conducive to mold growth and colonization of muscle surfaces. Dry-cured ham microbiota are generally appreciated, because their enzymatic activity contributes to the development of the characteristic flavor of this product. In fact, its flavor mainly depends on lipolysis and lipid oxidation *(7, 30),* proteolysis *(20),* and amino acid degradation *(5).*

The ham surface mycoflora, studied in different geographic areas, includes several species, mainly belonging to *Aspergillus, Penicillium,* and *Eurotium* genera *(10, 12, 24, 29, 36),* that are known to be able to produce mycotoxins under specific conditions. Because mycotoxins are secondary fungal metabolites that can cause acute and chronic health problems in humans and animals, further research on toxigenic mycoflora in meat products has been generated.

A survey conducted in Spain on dry-cured hams showed aspergilli as the dominant fungi, with around 50% of the samples contaminated by *A.* Section *Flavi.* These fungi are potential aflatoxin producers, but this metabolite has never, to our knowledge, been detected in monitored hams. After the artificial inoculation of ham pieces with *A.* Section *Flavi* isolates, the authors confirmed their potential ability to produce aflatoxins but not under the conditions commonly maintained in ham-ripening plants *(29).*

Ochratoxin A (OTA) has been detected by several authors in meat and meat products *(14, 17, 26, 28, 32).* OTA is a toxic compound *(2)* produced by different fungi, such as *Aspergillus ochraceus (15)* and *Aspergillus carbonarius (1),* among aspergilli, and *Penicillium verrucosum* and *P. nordicum* among penicillia. *P. nordicum* is a species isolated only from proteinaceous food, mainly meat products, such as sausages and hams *(18),* and it produces, under laboratory conditions, more OTA than *P. verrucosum* iso-

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lates; in this respect, the growth of *P. nordicum* on cured pork meat is a potential risk to consumers' health. Gareis *(16)* suggested that the presence of toxins in meat and meat products was the result of either direct contamination with molds or indirect transmission from animals exposed to naturally contaminated feed.

To verify the possibility of OTA direct contamination of ham, a study was carried out to (i) verify the presence of penicillia and aspergilli in ham-manufacturing plants, both in the air and on the ham, and (ii) characterize representative isolates for their in vitro ability to produce OTA.

MATERIALS AND METHODS

Manufacturing plants. Seven manufacturing plants (identified by the letters A through G), located in different areas of northern Italy, were considered in this study for air monitoring and ham sampling. Three plants were located in the countryside (A, E, and G), and four were in a village (B, C, D, and F); their working capacity varied between 65,000 and 320,000 hams produced per year. All plants were organized with separate rooms for ham drying, maturing, and aging; they were air conditioned, but it was common practice to open the windows on sunny days with mild temperatures and low air humidity.

In all monitored plants, the maturing phase was carried out for 3 to 4 months in maturing rooms (MRs). At the end of this period and after fat application, dry-cured hams were moved to aging rooms (ARs), where they were kept for 7 to 8 months, until the established overall processing time (14 months). Drying rooms were not monitored because of the short sojourn of hams in this area (1 week) in all plants. Drying, maturing, and aging make up the whole ripening stage of dry-cured hams.

Environmental data recording. Air temperatures and RH values were recorded in the MRs and ARs of each manufacturing plant, every 10 min, with a data logger (ESCORT Data Logging Systems Ltd., Atlanta, Ga.) over a 12-month period. Data were continuously collected in each plant, alternatively in MRs and ARs, with a 15-day cycle.

Air monitoring for fungal bioload. Petri dishes (diameter, 9 cm), with potato dextrose agar (infusion from potatoes, 200 g; dextrose, 15 g; agar, 20 g; and water, 1,000 g) as the medium, were exposed to monitor aerial fungal bioload. In each MR and AR of each plant, five dishes, properly labeled to identify plant, room, and replicate, were exposed for a 6-h period every 15 days for 12 months from July 2002 to June 2003.

After exposure, the plates were closed with their lids, sealed with Parafilm, and incubated at 25°C for 7 days in the dark; then, fungal colonies were identified to the genus level, on the basis of their morphological characters, and those belonging to penicillia and aspergilli were counted *(27, 28),* because they represent a potential risk for mycotoxin production *(22, 24, 35).*

Results were expressed as means of colonies per square centimeter of exposed surface. As a rule, when the colony numbers per plate became too numerous to count, they were considered equal to three colonies per square centimeter.

Ham sampling. Sixteen 14-month-old dry-cured hams were taken from each plant; four hams were randomly chosen four times during a 12-month period, with a 3-month interval. Two different kinds of samples were obtained from each ham: (i) a portion (4 by 4 by 0.5 cm [depth]) cut from the muscle surface near the skin boundary and (ii) the outer muscle (a portion of the outer part of muscle, taken from the upper skinless ham area, 1-cm depth).

Outer muscle samples were minced (1- to 2-mm diameter of particles) after fat removal. Portions were stored under vacuum, while minced muscles were frozen; all samples were analyzed within 10 days.

Portions were processed with a sterile scalpel; three 0.5-g subsamples were cut, put separately in petri dishes with Czapek yeast agar as the medium (sucrose, 30 g; yeast extract, 5 g; $NaNO_3$, 3 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; K_2HPO_4, 1 g; agar, 20 g; and water, 1,000 ml), and incubated at 25°C for 7 days in the dark. All fungi identified as aspergilli and penicillia, on the basis of their morphological characters, were counted. All isolates were subcultured, purified, and stored in the fungal collection of the Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore in Piacenza. The percentage of portions positive for penicillia was recorded.

Minced muscle samples were thawed at room temperature; a 0.3-g subsample was taken, extracted with 3 ml of bi-distilled sterile water, and shaken for 1 min with a mechanical shaker. Three aliquots of 1 ml of the suspension were separately plated in petri dishes with Czapek yeast agar as the medium and incubated at 25°C for 7 days. All fungi identified as aspergilli and penicillia, on the basis of their morphological characters, were counted and expressed as the number of CFU per gram. Representative isolates of penicillia were subcultured, purified, and stored in the above-mentioned fungal collection.

Fungal identification. Isolates belonging to the aspergilli were identified at the section level *(28),* while representative isolates of penicillia were identified at the species level. On the basis of macroscopic characters, isolates were selected, with an attempt to maintain their proportion in the population. Selected isolates were inoculated according to Pitt *(27),* with the support of a vial containing water agar (0.2%) on petri dishes with Czapek yeast agar as the medium, incubated at 25°C for 7 days, and observed for their macroscopic characters (colony morphology, upper and reverse color, and colony-colored contour), growth rate, and microscopic characters (shape and size of conidiophores and conidia). Thirteen species were identified, and an isolate from each species was confirmed by CABI Bioscience (Egham, UK). No distinction was made between *P. verrucosum* and *P. nordicum,* because this separation is reliable only if chemical or molecular techniques are used *(11, 18).* According to the literature, OTA-producing penicillia on meat products are considered *P. nordicum (6, 11, 18).*

OTA production by penicillia. Representative *Penicillium* isolates collected both from the air and the hams (around 2%) were tested for their OTA-producing ability, following the method suggested by Bràgulat et al. *(8).* Isolates were transferred to Czapek yeast agar plates and incubated at 25°C for 7 days in the dark; then, three plugs (0.46-cm diameter) were taken from each plate and dipped for 1 h in 1 ml of methanol. One hundred microliters of the filtrate was diluted with high-performance liquid chromatography (HPLC) mobile phase (900 μl), and the solution was then filtered with a Cameo 13N, 0.45-μm nylon syringe filter, (Micron Separations Inc., Westborough, Mass.) before HPLC analysis.

OTA determination by HPLC. OTA was purchased from Sigma (St. Louis, Mo.). An OTA solution (40 mg/ml in benzene: acetic acid [99:1, vol/vol]) was calibrated spectrophotometrically (Lambda 2, Perkin-Elmer Corp., Norwalk, Conn.) at 333 nm with an extinction coefficient of 5,550 *(3)* and stored at −20°C when

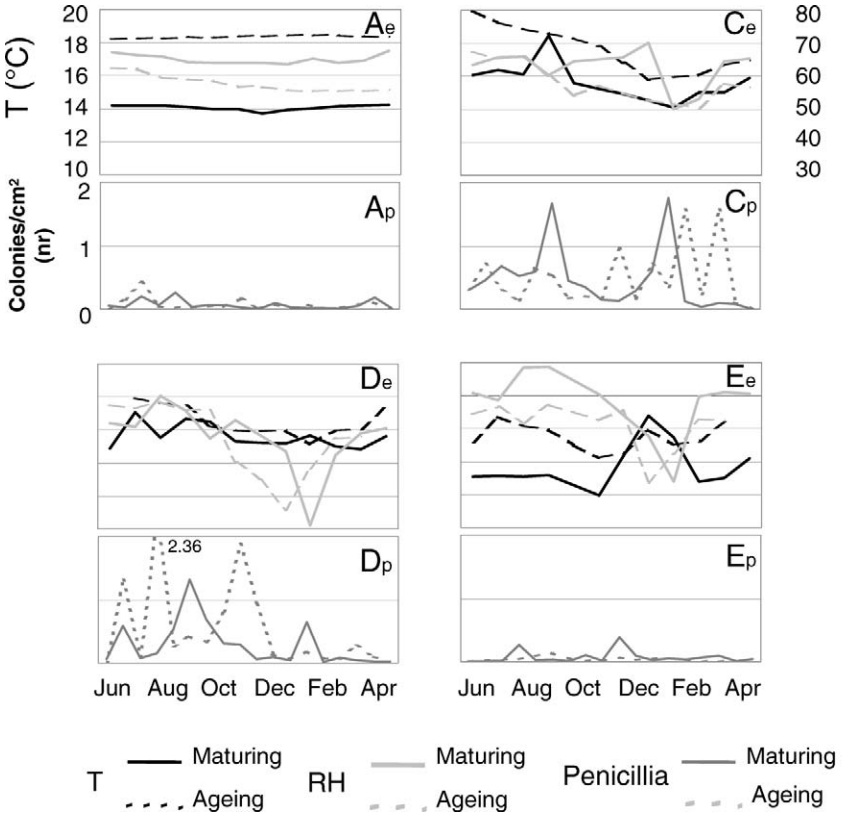

FIGURE 1. *Mean values of air temperature (T°C), % relative humidity (RH) (e), and fungal presence (colonies per square centimeter of exposed petri dishes) (p) measured in the maturing rooms (MR) and aging rooms (AR) of four dry-cured ham manufacturing plants during a 1-year survey (see "Materials and Methods" for details). Only plants showing more remarkable differences as to environmental and fungal data were displayed.*

not in use; after the OTA solution had been calibrated, working standards were prepared by evaporating an exact volume under a stream of nitrogen and redissolving the residue in the mobile phase.

The HPLC system consisted of a Perkin-Elmer 200 instrument equipped with an ISS (isotonic saline solution) 200 sampling system (loop volume, 150 μl) and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) set at 333-nm excitation and 470-nm emission. The system was controlled by Perkin-Elmer Turbochrom PC software. A Select B RP-8 column (5-μm particle size; inside diameter, 150 by 4 mm; Merck, Darmstadt, Germany) was employed at room temperature, with a mobile phase of acetonitrile:2% acetic acid in water (at 41:59 for OTA and at 55:45 for OTA methyl ester), at 1.2 ml/min. The injection volume was 30 μl.

OTA standards of 2 to 60 pg were injected. Turbochrom PC software was used for quantification on the basis of peak areas. For qualitative confirmation of positive samples, OTA was derivatized by methylation of the extracts, and HPLC analysis was subsequently carried out *(37).* The detection limit for OTA production was 0.5 μg per kg of medium.

Data analysis. Data analysis was carried out by the statistical package MSTAT-C (version 1, 1991; Michigan State University, East Lansing). An analysis of variance (ANOVA 1) was applied to environmental data. ANOVA model 13 (randomized complete block design for factor A, with factor B as a split plot on A and factor C as a split plot on B), was applied to data on the fungal presence in the air; model 9 (randomized complete block design for factor A, with factor B as a split plot on A) was applied to data on the fungal presence in the ham. Means were compared by the least significant difference test ($P < 0.05$).

A correlation analysis was run between the data on the fungal counts in the air and on the hams and environmental data.

RESULTS AND DISCUSSION

Environmental conditions of plants. Air temperature and RH values, recorded during the whole year of monitoring, are shown in Figure 1. Ripening rooms of some plants (mainly C and D for temperature and E for RH) proved to be influenced by external meteorological conditions, even if equipped with air conditioning. Temperatures in the ripening rooms varied between 11 and 21°C, registered in the MR of plant D in late February and in the AR of plant C in June, respectively. The RH varied between 30%, registered in the AR of plant F in late January, and 80%, reached in early August in the MR of plant E.

There were differences between plants as to their environmental condition management (Fig. 1). They can be summarized as follows. (i) Plant A showed remarkably steady environmental conditions during the whole year, characterized by intermediate RH values, low maturing, and high aging temperature. (ii) Plant B had low temperatures and high RH values (with the exception of the winter period), and the RH was the same in both the MR and the AR. (iii) Plants C, D, and F displayed a similar trend, characterized by intermediate RH and temperature values, with a decrease in plants D and F during the winter. (iv) Plant E functioned with high RH values and low temperatures during maturing. During the winter period, a sharp decrease in RH occurred. (v) Plant G was characterized by low RH during maturing and aging and by a high temperature during the spring in aging.

Environmental data recorded during the whole year in the plants were compared (Table 1), and differences ($P < 0.05$) were detected among plants and between maturing

TABLE 1. *Results of a one-way ANOVA applied to temperature, relative humidity, and number of penicillia detected in maturing (MR) and aging (AR) rooms in seven dry-cured ham manufacturing plants monitored between July 2002 and June 2003 and considered in the study*[a]

	Temp (°C)		Relative humidity (%)		Penicillia (colonies/cm^2)	
Plants	MR	AR	MR	AR	MR	AR
A	14.1 BX	18.3 AZ	64.8 AY	57.7 BXW	0.07 AX	0.07 AX
B	12.6 BV	13.8 AV	66.1 AZY	67.0 AZ	0.08 AX	0.06 AX
C	15.8 BY	17.6 AZ	63.2 AX	58.4 BX	0.49 AZ	0.51 AZ
D	15.7 BY	16.5 AY	58.9 AW	56.2 BW	0.28 BZ	0.52 AZ
E	13.6 BW	15.4 AW	66.3 AZ	61.8 BY	0.07 AX	0.04 AX
F	16.5 AZ	15.7 BX	65.0 AZY	52.1 BY	0.18 AY	0.16 AX
G	16.4 BZ	17.6 AZ	54.8 AY	57.7 BXW	0.04 BX	0.28 AY
Mean	**14.9 B**	**16.3 A**	**62.4 A**	**57.6 B**	**0.17 B**	**0.24 A**

[a] Different letters (A and B) for the two groups of data (maturing and aging) indicate significant differences between the two ripening rooms ($P < 0.05$) for the single plant and the mean value (bold); the other set of different letters (V through Z) indicate significant differences among plants within each ripening room ($P < 0.05$, multiple paired *t* test according to the least significant difference test).

and aging phases. In both phases, plants B and E were regarded as the coldest and wettest, and plant G was the warmest and driest; other plants displayed variable values during the maturing and aging phases.

The maturing phase was carried out in conditioned rooms, working at higher RH levels and lower temperatures ($P < 0.05$) if compared to the next aging phase (Table 1); this choice was aimed at preventing an excessive drying and crusting of the exposed ham muscle surfaces. During the aging period, fat application on the unskinned muscle sections of dry-cured hams allows the outer muscle layers to rehydrate and the room RH to decrease.

An average tendency to lower RH values inside the ripening rooms was found if compared with the maturing and aging data reported by Spotti et al. *(35)* and Parolari *(25)*. This trend could be ascribed as an attempt to prevent process drawbacks, such as poor weight losses during the mid-ripening period, uncontrolled mold and mite growth on the ham surface, and the onset of surface spoilage due to the slow drop in surface water activity.

Fungal presence in the air. A total of 18,200 penicillia and 1,067 aspergilli were counted on exposed dishes; penicillia represented 94% of the relevant fungi counted. These results concur with reports by other authors *(12, 34)*. Results obtained from the ANOVA showed that all of the factors considered (plant, stage, and sampling time) influenced the counts of penicillia in the air as well as their interaction ($P < 0.01$; data not shown). In particular, significant differences were found among plants (Table 1), with plant C being the most contaminated and plants A, B, and E being the least contaminated. Air in the ARs resulted in significantly more contamination than air in the MRs (0.24 versus 0.17 isolates per cm^2, respectively), while sampling dates did not show a clear trend.

Maturing rooms. The counts of penicillia largely exceeded those of aspergilli in all manufacturing plants, except in E. These results concur with those of Comi et al. *(12),* who reported that the genus *Aspergillus* was not predominant in dry-cured ham-ripening rooms. Plants C and D had the highest *Penicillium* counts, while plant F showed an intermediate count among the tested factories (Table 1). These plants had conditioned rooms set at medium-high temperature and intermediate RH values, when compared with the others, as shown in Figure 1 and Table 1. Plants A, B, and E (low temperature) and plant G (low RH) yielded the lowest counts. In particular, in plants A and G, the temperature and RH values were maintained at the same level during the whole year of monitoring.

Aging rooms. No favorable effect of temperature increase during aging was found on aspergilli, which were almost absent.

The large presence of penicillia in plants C and D detected in the MR was confirmed. In plant G, these fungi increased, possibly because of the temperature increase recorded in this factory during the aging process (Table 1). Likewise, plants A, B, E, and F showed the lowest *Penicillium* count during aging, probably because of the lower temperatures recorded.

Plants B and E worked at the highest RH and lowest temperature in both phases (with the exception of wintertime) and achieved low counts for penicillia, even though there was abundant growth of other molds on the ham surface (data not shown).

The average count of penicillia in the AR increased when compared to the MR; possibly the temperature increase of the AR (Table 1) favored *Penicillium* presence in the air, while the lowering of the RH, with respect to the maturing phase, seemed to have little effect on this fungal population. As to the factories with a high penicillia count, plant D had a sharp drop (below 40%) in the RH values of the ripening rooms (Fig. 1) during the winter months (December through February) and showed a corresponding decrease in penicillia counts. Plant F in the AR displayed a similar trend. In plant C, where the average RH values were always above 50%, the large presence of *Penicillium* in the air of the ripening rooms did not show any clear decrease during the monitored period.

No significant correlation was found between the temperature or RH and the fungal presence in the air in either the MR or the AR.

Ham sampling: portions. Although meat products were found to be a suitable substrate for *A. ochraceus* growth and OTA production *(9, 14),* aspergilli were rarely isolated from the portions of muscle surface analyzed, while penicillia were isolated frequently, which supports the data reported for fungi in the air. The percentages of portions positive for penicillia, expressed as average values from each ripening plant, were between 38 and 90% and were found in plants B and C, respectively (Fig. 2). The

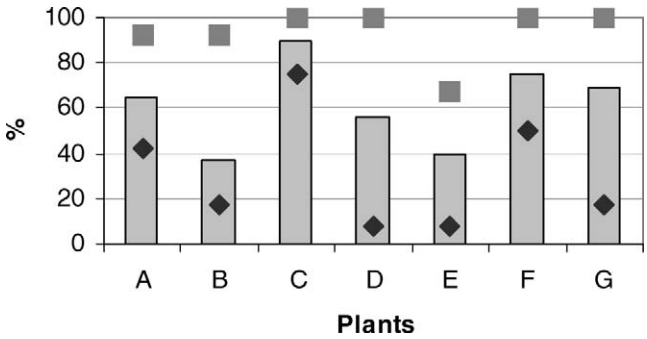

FIGURE 2. *Mean percentage and minimum and maximum values (◆, min; □, max) of portions positive for penicillia.*

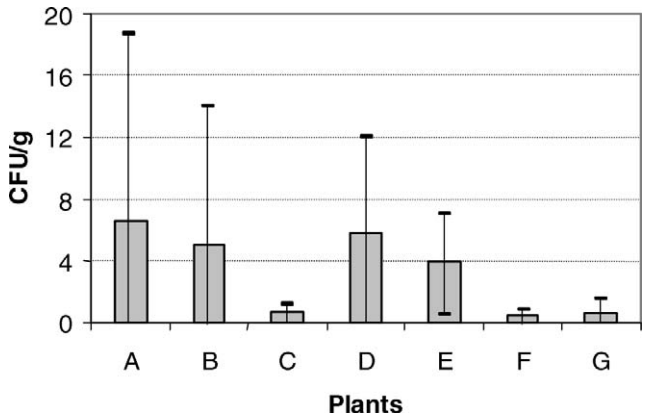

FIGURE 3. *Mean number and confidence interval of CFU per gram found in the outer layer of dry-cured hams.*

incidence of portions contaminated by penicillia showed a large variability with respect to results coming from the same plant, possibly because of the environmental conditions during the sampling periods, whereas a positive relationship was found with the fungi isolated from the air.

Ham sampling: muscle. Aspergilli were never isolated in muscle samples. Penicillia were detected in all manufacturing plants (Fig. 3), but the number of CFU per gram was low. An ANOVA was applied, and the only significant effect highlighted was due to the sampling time, with the second sampling between August and October having a significantly higher contamination ($P = 0.01$; 5.2 versus 1.0 colonies per g).

Fungal identification and characterization. One thousand ninety-two aspergilli isolates were identified; 1,067 were collected from the air, 25 from the ham portions, and none from the muscle. Seventy-six isolates from the air (7%) and all those obtained from the ham were identified at the section level, with sections *Nigri, Circumdati,* and *Flavi* being the most common. Isolates from the air were equally shared between sections *Nigri* and *Circumdati,* with only three isolates attributed to *Flavi.* Of the 19,811 identified penicillia isolates, 18,200 were from the air and 1,611 were from the ham; 231 isolates collected from the air and 76 from the ham samples were identified at the species level and included in the fungal collection of the Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore. In the air, the most represented species (Table 2) was *P. nalgiovense* (58% of the total penicillia), followed by *P. nordicum* (10%), *Penicillium brevicompactum* (8%), and *Penicillium chrysogenum* (5%). Other species were detected, but their incidence was lower than 5%. *P. nordicum* was found in all plants and rooms tested. Fifty percent of *P. nordicum* isolates were able to produce OTA when tested in vitro. In particular, 32% of the positive strains were able to produce less than 100 ng of OTA per g, 26% were able to produce from 100 to 1,000 ng of OTA per g, and 42% were able to produce more than 1,000 ng of OTA per g.

P. nalgiovense was also the most common species (64% of the total penicillia) in the ham samples, followed by *P. nordicum* (26%), with 50% of the isolates able to produce OTA in vitro; a few other penicillia were detected, but rarely. The percentage of *Penicillium* isolates regarded as toxigenic in their ability to produce OTA is in agreement with the findings of Leistner and Eckardt *(19)* for *Penicillium* isolates originating from meat products. However, they differ from the results obtained by Comi et al. *(12),* who tested several *Penicillium* isolates from Istrian ham in vitro without finding any toxic metabolites.

Potential risk of OTA formation during dry-cured ham manufacture. Potential OTA producers, such as *P. nordicum,* are commonly present in ham manufacturing plants and, with limited concentration, in hams.

This presence can explain the OTA contamination found in some surveys on dry-cured ham samples *(14).* Surveys carried out in Italy showed a low OTA content *(33),* but recently, Pietri et al. *(26)* found 5 of 30 analyzed samples with OTA levels exceeding 1 μg/kg, the limit set by the Italian Ministry of Health Guideline *(21).*

These findings suggest that it is necessary to conduct further studies to better understand the ecological conditions favorable to fungal growth and OTA production, particularly those relevant to penicillia metabolism *(23),* to fur-

TABLE 2. *Species of penicillia and number of isolates obtained from the air and the hams of the seven dry-cured ham manufacturing plants included in the fungal collection of the Institute of Entomology and Plant Pathology, Universitá Cattolica del Sacro Cuore, and taken into account in the study*

Penicillium species	Air	Ham portion	Ham muscle
aurautiogriseum	1	—[a]	—
brevicompactum	18	—	—
chrysogenum	12	—	2
citrinum	10	3	1
crustosum	6	—	—
expansum	9	—	—
glabrum	1	—	—
glaudicola	1	—	—
griseofulvum	1	—	—
nalgiovense	134	28	21
nordicum	24	9	11
oxalicum	7	—	—
roquefortii	7	—	1

[a] —, not found.

nish precise information to ham producers. Additionally, more studies are needed of the chemical profiles of penicillia isolated on ham and in the plants that manufacture it because, as reported by several authors *(4, 31, 36),* they are able to produce metabolites other than OTA.

More knowledge about OTA is imperative because the proposals from the Commission of European Communities *(13)* require the legal limits for OTA presence in meat products to be fixed as soon as possible.

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