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Salting of dry-cured meat – A potential cause of contamination with the ochratoxin A-producing species *Penicillium nordicum*

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ABSTRACT

Penicillium nordicum is a known contaminant of protein-rich foods and is primarily found on dry-cured meat products. It is an important producer of the mycotoxin ochratoxin A, which has nephrotoxic and cancerogenic activities. Recently a high number of *P. nordicum* strains was isolated from different dry-cured meat products from one of the Slovenian meat-processing plants. Since we have isolated *P. nordicum* in high counts also from Artic habitats, such as sea water and sea ice and due to its ability to grow well at low temperatures and at increased salinity, sea salt was suspected as the possible source of *P. nordicum*. In the present study contamination of meat products, air in the meat-processing plant and sea salt used for salting were analysed. When 50 g of salt sample from a sealed package was dissolved in sterile water and filtered, 12 colonies of *P. nordicum* were obtained on solid medium incubated at 15 °C, while a salt sample from an open vessel in the meat-processing area developed high, uncountable number of colonies. Amplified fragment length polymorphism analyses of *P. nordicum* isolates from different sources showed that contamination of meat products via salt was possible. Three selected isolates examined for extrolites all produced ochratoxin A. As contamination of dry-cured meat products with *P. nordicum* represents a potential health risk for consumers and workers in the meat-processing plants, salt should be taken into account as a potential cause of such contaminations.

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1. Introduction

The mould *Penicillium nordicum* is a widely distributed contaminant of protein-rich food. It is most often found on dry-cured meat products (cured ham, salami), salted fish and, occasionally, on cheese and jam. It can grow well at low temperatures (15 °C) and increased salinity (5% NaCl), and therefore it is often isolated from refrigerated and salted proteinaceous food products (Frisvad and Samson, 2004; Samson et al., 2002). It is an important and consistent producer of ochratoxin A (OTA), which can be detected in the *P. nordicum* mycelia and spores, and also in food products that *P. nordicum* contaminates (Frisvad and Samson, 2004). OTA is a potent mycotoxin, and based on toxicological data obtained with experimental animals, it has nephrotoxic, nephrocarcinogenic, teratogenic, neurotoxic and immunotoxic activities (EFSA, 2006; Marin-Kuan et al., 2008; Van Egmond, 2002). OTA is involved in porcine and chicken nephropathy (Stoev et al., 2010) and is suspected to be one of the most important aetiological factors in the human Balkan endemic nephropathy and in the occurrence of tumours of the urogenital tract. However, the epidemiological data are not at present adequate, and thus these associations have not yet been unequivocally confirmed (EFSA, 2006; Grollman et al., 2007; Mally et al., 2007; Marin-Kuan et al., 2008; Pfohl-Leszkowicz, 2009; Stoev et al., 2010).

Within the genus *Penicillium*, *P. nordicum* and *P. verrucosum* are the only known OTA producers. These two species are phylogenetically related, and they have very similar morphological characteristics; however, they have different ecological preferences (Larsen et al., 2001; Lund and Frisvad, 2003). *P. verrucosum* is primarily found on cereals, and is therefore responsible for the major contribution to human OTA exposure via cereals and cereal products (Frisvad and Thrane, 2002; Lund and Frisvad, 2003). *P. nordicum* is the most important OTA-producing species isolated from dry-cured meats (Frisvad and Thrane, 2002; Samson et al., 2002), therefore it is crucial to prevent contamination of meat with this species, so as to guarantee the safety of dry-cured meat products. *P. nordicum* strains have been isolated also from the air from meat-processing plants (Battilani et al., 2007; Bogs et al., 2006; Dragoni and Cantoni, 1979; Dragoni and Marino, 1979),

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while other possible sources of *P. nordicum* contamination and its occurrence in nature remain unknown.

In summer 2006 *P. nordicum* caused extensive contamination of dry-cured meat products in one of the Slovenian meat-processing plants and a very high number of *P. nordicum* isolates were obtained from three dry-cured meat products (Sonjak et al., 2010). Contamination with *P. nordicum* was observed for the first time in this meat-processing plant, therefore it was important to determine its cause. In studies of mycobiota from meat-processing areas, the air and equipment surfaces are rarely investigated, as are the raw materials, such as spices, meat and fat (Asefa et al., 2010; Mižáková et al., 2002; Sørensen et al., 2008). In this study contamination of various environments in meat-processing plant with *P. nordicum* was evaluated. To identify closely related *P. nordicum* isolates with a presumed common origin and to relate them to possible points of contamination, the *P. nordicum* isolates obtained from dry-cured meats, air and salt samples were analysed using the amplified fragment length polymorphism (AFLP) fingerprinting technique, which enables the detection of polymorphisms in the genome at low taxonomic ranks (Savelkoul et al., 1999; Vos et al., 1995).

2. Materials and methods

2.1. Monitoring of fungi

Five items of each of the three dry-cured meat products: dry-cured pork neck in a casing (product 1), smoked dry-cured ham (product 2), and a dry sausage in a casing (a salami; product 3) were sampled on five occasions at intervals of about 10 days from July to September 2006. By using malt extract agar (MEA; Raper and Thom, 1949) and MEA with 5% NaCl (MEA5%NaCl, Gunde-Cimerman et al., 2003; Samson et al., 2002), both containing 50 mg l^{-1} chloramphenicol (cm), a high number of *P. nordicum* isolates were obtained from meat outer surfaces (Sonjak et al., 2010). Of these isolates five – seven isolates from at least three different items of each dry-cured meat product were selected for the present study to perform AFLP analysis. Furthermore one isolate from each product was included in HPLC analysis and one isolate from product 1 and one from product 2 in sequencing (Table 1).

For this study also air in the meat-processing plant and salt used for curing the meat were sampled once in September 2006. Air was sampled by exposing sterile MEA5%NaCl+cm plates for 10 min: in the production area; in the drying chamber of product 1 (temperature 14–21 °C, controlled relative humidity); and in the ripening chamber of product 2 (temperature 16–18 °C, controlled relative humidity). The salt was sampled from the sealed packages and the open vessels in the production area. For each salt sample, 50 g was aseptically weighted and dissolved in 150 ml sterile distilled H_2O and passed through 0.45 μm membrane filters (Millipore), which were then aseptically transferred to MEA5% NaCl+cm medium. For the control 200 g of sea salt of other salt producer not used in this meat-processing plant was also analysed. All of the medium plates were incubated at 15 °C for 10 days in the dark. After incubation, the mycelia of *P. nordicum*-like colonies: white with brown reverse (Fig. 1), were transferred to MEA medium to obtain pure cultures. The isolated fungi were preserved in silica gel and are maintained in the EX-F Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia.

2.2. Morphological characterisation

For the macro-morphological observations, most of the isolates were three-point inoculated on MEA, Czapek yeast autolysate agar (CYA), yeast extract sucrose agar (YES), and creatine sucrose agar (CREA), and grown at 25 °C for 7 days in the dark (Frisvad and Samson, 2004). For the determination of the micro-morphological characteristics, microscope slides were prepared for the selected isolates from the MEA medium. A water solution of 60% (v/v) lactic acid without a colour dye was used as the mounting medium. The slides were examined by differential interference contrast microscopy under oil immersion at up to 1000× magnification with a BX51 microscope (Olympus). Digital micrographs were taken with a DP12 digital camera and analysed using the DPSOFT 3.2 application software (Olympus).

Table 1
List of *Penicillium nordicum* isolates used for the AFLP analysis, their source of isolation and AFLP pattern number. GenBank accession numbers and extrolite profiles are given for the selected isolates.

EX-F number	Source	AFLP pattern	GenBank accession number (similarity with *P. nordicum*)	Extrolite profile
2795	Product 1, item 4	p1		Ochratoxin A Verrucolone Anacine
3198	Salt from sealed package	p1		
3003	Product 1, item 2	p2	HM103380 100% (2e-166)	
2823	Product 1, item 4	p2		
3004	Product 1, item 5	p2		
3005	Product 1, item 5	p2		
2792	Product 2, item 1	p2	HM103379 99% (e-value 0)	Ochratoxin A Verrucolone Sclerotigenin
2989	Product 2, item 5	p2		
3201	Air from drying chamber of product 1	p2		
3202	Air from drying chamber of product 1	p2		
3206	Air from production room	p2		
3210	Salt from open vessel	p2		
3197	Salt from sealed package	p2		
2960	Product 1, item 1	p3		
2962	Product 1, item 1	p3		
3046	Product 2, item 3	p3		
2961	Product 2, item 4	p3		
3027	Product 2, item 4	p3		
2956	Product 3, item 1	p3		
3033	Product 3, item 1	p3		
2790	Product 3, item 2	p3		Ochratoxin A Verrucolone Anacine Sclerotigenin
3030	Product 3, item 2	p3		
3031	Product 3, item 4	p3		
3208	Air from drying chamber of product 1	p3		
3196	Salt from sealed package	p3		

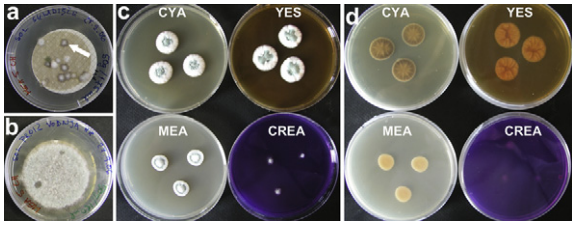

Fig. 1. (a, b) Fungal growth obtained after filtering dissolved salt samples and incubation of Agar plates with membrane filters; (a) Salt sample from an original, sealed package; white arrow indicates the *Penicillium nordicum* colony; and (b) salt sample from an open vessel. **(c, d)** Morphology of *P. nordicum* on the four different identification media: CYA, YES, MEA and CREA, as obverse (c) and reverse (d) views.

2.3. Determination of production of ochratoxin A and other extrolites

Selected isolates (Table 1) were three-point inoculated on CYA and YES media, and grown at 25 °C for 7 days in the dark. Isolate EX-F 2790 was additionally three-point inoculated on CYA medium with 5, 10, 15 or 20% NaCl added and grown at 25 °C for 21 days (5%, 10% and 15%), 32 days (15%) or 50 days (20%) in the dark. After incubation three agar plugs (6 mm in diameter) were cut from different parts of the colony from each medium. Plugs were then transferred into 500 µl of the solvent mixture, methanol-dichloromethane-ethyl acetate (3:2:1), containing 0.5% (v/v) formic acid, and then extracted ultrasonically for 60 min. The organic phase was transferred to clean vial and evaporated to dryness during centrifugation under a vacuum. The residue was redissolved in 500 µl methanol, filtered through 0.45 µm Minisart RC4 filter (Sartorius), and analysed by high-performance liquid chromatograph (HPLC; system A1100; Agilent) (Frisvad and Thrane, 1987, 1993; Smedsgaard, 1997). The separation was obtained on a 2 × 100-mm Luna2 OOD-4251-BO-C18 column (Phenomenex), with a C18 precolumn, both packed with 3 µm particles. The column was maintained at 40 °C. Sample injection volume was 5 µl. The elution gradient was initially linear, starting from 85% water/15% acetonitrile, and going to 100% acetonitrile over 20 min, which was then maintained for 5 min. A flow-rate of 0.4 ml min^{-1} was used. Both eluents contained 0.005% (v/v) trifluoroacetic acid. An alkylphenone analytical standard was used. Extrolites were identified by comparison with standards and by their characteristic spectra. The ochratoxins were measured with fluorescence detection (excitation: 230 nm, emission: 450 nm), presenting peak height in luminescence units, while all other metabolites were measured by UV detection at 210 nm, using peak areas in logarithmic units.

2.4. Sequencing

Partial *β-tubulin* gene sequences were obtained for the selected isolates (Table 1). Their DNA was first extracted by mechanical lysis (Gerrits van den Ende and de Hoog, 1999) from ~200 mg of cultures grown on complete yeast extract medium (CYM) (Raper et al., 1972) at 25 °C for 5 days in the dark. Amplification of the partial *β-tubulin* gene was performed in a thermal cycler (Mastercycler® epgradient, Eppendorf) and carried out as described by O'Donnell and Cigelnik (1997), using the T1 and T22 primers. The PCR products were checked electrophoretically on 1% agarose gels. DNA purification and automated DNA sequencing was provided by the Macrogen Company (Seoul, Korea). The partial *β-tubulin* gene sequences obtained were compared to sequences available from the National Center for Biotechnology Information (NCBI), using the BLAST-n programme (Altschul et al., 1990, 1997). The sequences were aligned with ClustalW (Thompson et al., 1994) using the MEGA4.0 software (Tamura et al., 2007). The partial *β-tubulin* sequences generated in the present study were deposited at GenBank, and their accession numbers are given in Table 1.

2.5. AFLP fingerprinting

AFLP analysis was performed for the selected isolates (Table 1) using the protocol described by Vos et al. (1995), and modified by Radišek et al. (2003) and Sonjak et al. (2007). Furthermore, the final reaction volumes were also modified here: the genomic DNA (250 ng) was double digested with *Eco*RI (1.25 U) and *Msp*I (1.25 U) in a final volume of 20 l. The restriction fragments were then ligated with 2.5 pmol of *Eco*RI adaptor and 25 pmol of *Msp*I adaptor using 0.3 U of T4 DNA ligase, in 5 l. Preamplification of 2.5 l of the restriction–ligation mix was performed with 37.5 ng of each of *Eco*RI and *Msp*I primers with no selective nucleotides, in a final volume of 25 l. In the second selective amplification of 2 l of preamplified DNA, primer pair *Eco*RI+AC (15 ng; fluorescently labelled with Cy5) and *Msp*I+TA (15 ng), which gave high-quality and informative chromatograms in our previous study (Sonjak et al., 2007), was used in a final volume of 10 l. The reagents used were from Fermentas. All of the PCR reactions were carried out in a thermal cycler (Mastercycler® epgradient, Eppendorf). Reproducibility of the AFLP profiles was confirmed by performing the whole procedure on two independent DNA preparations for each of two isolates.

The final products were denaturated and separated by polyacrilamide gel electrophoresis using ReproGel™ (Amersham Biosciences) and an ALFexpress II DNA Analyser system (Amersham Pharmacia biotech). The gel was run for 450 min at 55 °C, 1500 V, 60 mA and 15 W, and with sampling intervals of 1 s. The 50–500 ALFexpress sizer (Amersham Biosciences) was used as an external standard. Fragment analysis was performed with the ALFwin Fragment Analyser 1.00 software. The chromatograms were visually scored for both polymorphic and monomorphic fragment peaks; each peak was treated as a unit character and scored for presence or absence.

3. Results

3.1. Isolation of P. nordicum

Air analyses for the presence of *P. nordicum* in the production area and in the drying chamber of product 1 resulted in one and four *P. nordicum* isolates, respectively; the plate exposed to air in the ripening chamber of product 2 was overgrown and was not further analysed. Surprisingly, *P. nordicum* was also isolated from the salt used for the salting of the meat in the first production step. Twelve of the colonies isolated on solid medium from the 50 g of salt from the sealed packages were identified as *P. nordicum*; plating of the same quantity of salt obtained from the open vessel in the production area resulted in plates that were completely overgrown with mycelia identified as *P. nordicum* (Fig. 1). From the control sea salt sample *P. nordicum* was not isolated.

3.2. Identification

The macro- and micro-morphological characteristics of the *P. nordicum* isolates were in agreement with the species description (Frisvad and Samson, 2004). The colonies had a velutinous to floccose texture, and average diameters of 17, 24, 13 and 9 mm on CYA, YES, MEA and CREA media, respectively. Weak growth and no acid or base production were observed on CREA medium. On CYA medium, clear to light yellow exudate droplets were frequently observed. The reverse colour on CYA was cream to brown, and on YES it was cream yellow. The isolates had terverticillate conidiophores, and produced green-coloured, smooth-walled, globose to subglobose, 3.2-µm (median) conidia on MEA medium. The median dimensions of phialides, metulae and rami were 8.5 × 2.7, 12 × 3, and 17 × 3.3 µm, respectively. Analysis of the two partial *β-tubulin* gene sequences showed very high similarity (99–100%) with *P. nordicum* sequences from GenBank (Table 1). The accuracy of identification of *P. nordicum* isolates isolated also from dry-cured meat (Sonjak et al., 2010) was thus confirmed.

3.3. Extrolite production

Extrolite analysis of the three selected isolates showed that they produced ochratoxin A and verrucolone, and additionally anacine and/or sclerotigenin (Table 1), which is in agreement with the description of *P. nordicum* species (Frisvad and Samson, 2004). Additional analysis of the isolate grown on CYA with NaCl showed

that production of OTA was high at 5% NaCl but it was drastically reduced at 10% NaCl or more (Table 2). At 15 or 20% NaCl extrolites sclerotigenin and anacine were not detected. The prolonged incubation times had a positive effect on detection of OTα and interestingly its highest amount was detected at 20% NaCl.

3.4. AFLP

Twenty-five *P. nordicum* isolates derived from the three different dry-cured meat products (Sonjak et al., 2010), from the salt used in their production, and from the air of the meat-processing plant were analysed by AFLP (Table 1). Three different AFLP patterns, consisting of six polymorphic bands of 26 bands overall, with molecular sizes ranging from 50 to 500 bp (Fig. 2), were obtained. The AFLP results thus demonstrated: two isolates with pattern 1, one from product 1 and one from the salt; eleven isolates with pattern 2, four from product 1, two from product 2, three from the air, and two from the salt; and twelve isolates with pattern 3, two from product 1, three from product 2, five from product 3, one from the air and one from the salt (Fig. 2). All three of the patterns included isolates from the originally seal-packed salt.

4. Discussion

P. nordicum is a mycotoxinogenic food contaminant, which produces, in particular, large amounts of the nephrotoxic and cancerogenic mycotoxin OTA (Frisvad and Samson, 2004; Lund and Frisvad, 2003). Its heavy contamination of food, as found in the Slovenian meat-processing plant (Sonjak et al., 2010) can represent a potential food safety hazard.

P. nordicum has also been reported to occur relatively frequently on dry-cured meat products in Italy. It has been isolated from several dry-cured ham and dry-sausage processing plants (Battilani et al., 2007; Bogs et al., 2006; Iacumin et al., 2009), indicating that it is generally present in this area or present in the raw materials used. On the other hand, studies that have investigated mycobiota of dry-cured meat products from seven Norwegian (Asefa et al., 2009) and north European (Sørensen et al., 2008) meat-processing plants, and from Greek (Papagianni et al., 2007) and Spanish (López-Díaz et al., 2001) fermented sausage samples, have not shown the presence of *P. nordicum*, although the genus *Penicillium* was reported as the prevailing contaminant. Some of *P. nordicum* cultures have white conidia, including the type culture of *P. nordicum*, and are thus difficult to detect by the naked eye among *Penicillium nalgiovense* isolates, that also often have white conidia (Dragoni and Cantoni, 1979; Dragoni and Marino, 1979; Frisvad and Samson, 2004). Comi et al. (2004) carried out an investigation of moulds growing on dry-cured ham produced in Istria, close to Slovenia. Although these hams are covered with a mould coat that gives them their characteristic taste and appearance, out of 81 hams investigated from three different producers, strains of *P. nordicum* were not isolated.

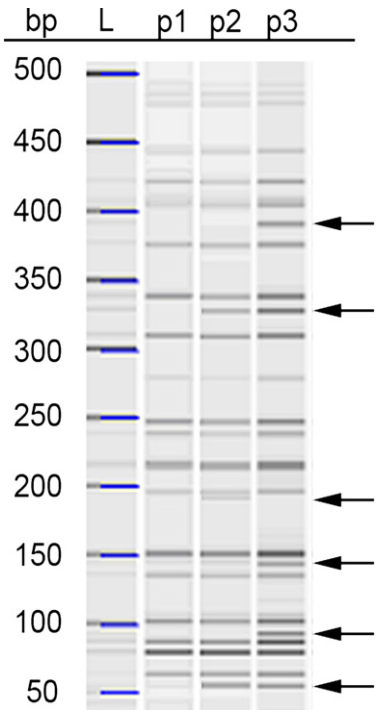

Fig. 2. The three different AFLP patterns obtained, marked as: p1, p2 and p3.The arrows indicate the polymorphic bands. L, ladder.

With the assumption that species identifications were accurate in the above mentioned studies than this relatively low occurrence of *P. nordicum* on dry-cured meat products in the various European plants investigated that differ in climate and geographical locations indicates that the potential source(s) of contamination with *P. nordicum* show a local presence in the Slovenian/north Italy area, or that the contamination could be due to the particular raw material(s) used and/or steps in the production.

Air can be an important source of contamination with conidium-forming fungi, and thus in the production of dried meats, the mycobiota in the indoor air is the main source of different moulds when starter cultures are not used (Scholte et al., 2002). The present study and the study by Battilani et al. (2007) have shown that *P. nordicum* can indeed be isolated from the air of dry-cured meat-processing plants. Although the *P. nordicum* conidia might have been transported by air from elsewhere, they were more probably released from the very local reservoir – the dry-cured meat surfaces that were covered with sporulating moulds.

P. nordicum is well known for its psychrotolerance and good growth at low temperatures. It also grows well at low water activities due to increased salt concentrations in the media (Frisvad and Samson, 2004). Indeed, we have tested the growth of *P. nordicum* on CYA media with different concentrations of NaCl at

Table 2
Extrolites produced by *Penicillium nordicum* isolate EX-F 2790 when grown on CYA medium with different concentrations of NaCl for 21–50 days at 25 °C in the dark.

Growth variable		Extrolite detected							
NaCl (%)	Days	OTA	OTB	OTα	Verrucolone	Sclerotigenin	Anacine	Indolalkaloids	
5	21	47	6	2.5	2.21	2.58	2.78	1.97	1.94
10	21	8	0	0	2.50	2.23	2.29	2.00	1.98
15	21	6.5	1.2	0	2.42	0	0	2.06	1.87
15	32	6	0.5	1.2	2.55	0	0	2.19	2.00
20	50	1	0.1	12.5	2.09	0	0	1.91	1.78

25 °C for 3 weeks, and growth was seen to occur also on medium with 20% NaCl.

Although little is known about *P. nordicum* ecology in temperate climates, it is interesting to note that recently *P. nordicum* has been isolated in large numbers from different Arctic habitats, such as sea water, glacial ice, and in particular, sea ice. All of these ecological niches have in common: low temperatures, low water activities and increased salinities (Gunde-Cimerman et al., 2003; Sonjak et al., 2006). These ecological data thus indicate a potential cause of contamination with *P. nordicum* – the sea salt used for the meat curing, followed by enrichment due to storage at low temperature (15 °C) in the dry-cured meat-processing plant. Indeed, several *P. nordicum* colonies were obtained on MEA with 5% NaCl agar plates incubated at 15 °C from salt samples from sealed packages, and even more so from the salt kept in open vessels within the meat-handling areas where is ready for the salting of the meat in the early steps of the process. This possible contamination via the salt was also supported by AFLP analysis, which was performed on selected isolates. Three different AFLP patterns were obtained, and each appeared for the *P. nordicum* strains isolated from both the meat samples as well as from the salt from sealed package. On the assumption that identical patterns reflect a shared clonal origin, we can conclude that at least three different clones were isolated from the dry-cured meats, and that each possibly originated from the salt, and was secondarily spread from the meat products into the air. It is worth noted that sea salt usually contains very low number of viable fungal spores and viable spores of *P. nordicum* were not recovered from 200 g of control sea salt sample obtained from another producer. The selection and use of salt with higher quality, as obtained with better controlled production, storage and transportation conditions, is therefore recommended.

Selected isolates originating from meat products produced different HPLC profiles of extrolites; however, all three produced OTA and verrucolone. The results of extrolite production on CYA medium with added NaCl were in accordance with those obtained by Battilani et al. (2010) and Geisen (2004) as production of OTA was high at 5% but it was drastically reduced at 10% or more NaCl added. We however additionally showed the reduction in the production of OTB, Sclerotigenin and Anacine at higher NaCl concentrations, whereas the production of OTα was, interestingly, increased at 20% NaCl. Battilani et al. (2010) and Geisen (2004) also showed that OTA production is drastically reduced at temperatures below 18 °C. If OTA is detected in dry-cured meat products, its concentrations rarely exceed 1.0 g kg^{-1} (Chiavaro et al., 2002; EFSA, 2006; Pietri et al., 2006). Higher OTA concentrations can be present on casings of sausages (Iacumin et al., 2009) or possible in dry-cured meat products with longer processing stages at high water activity and temperatures above 18 °C, as these conditions were shown to enhance OTA production in dry-cured meat model systems (Battilani et al., 2010). Since *P. nordicum* grows and sporulates very actively at temperatures around 15 °C, a lot of conidia can be released into the air from contaminated material and can be spread around a meat-processing plant via air, representing an additional source of human exposure to OTA that should not be overlooked (Duarte et al., 2009).

We can conclude that mycotoxin OTA-producing *P. nordicum* is present on dry-cured meat processed in certain parts of Europe, and that possible points of contamination could be the raw material(s) used in the production. We have shown that the sea salt used for salting meat in the early stages of dry-cured meat processing can be a source of *P. nordicum*, which can then grow and proliferate very well on dried meats, and is secondarily spread into the air. Contamination of dry-cured meat products with *P. nordicum* thus represents a potential health hazard for consumers, and for the workers in the dry-cured meat-processing plants; therefore any source(s) of contamination should be identified and withdrawn from the production, or replaced by safe, non-contaminated material(s).

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