Food Microbiology 26 (2009) 65–70

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Moulds and ochratoxin A on surfaces of artisanal and industrial dry sausages

Lucilla Iacumin [a], Luca Chiesa [b], Daria Boscolo [c], Marisa Manzano [a], Carlo Cantoni [b], Sandi Orlic [d], Giuseppe Comi [a,*]

[a] Dipartimento di Scienze degli Alimenti, Facoltà di Agraria, Università degli Studi di Udine, via Sondrio 2, 33100 Udine, Italy
[b] Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza degli Alimenti, Facoltà di Medicina Veterinaria, Università degli Studi di Milano, via Celoria 12, 20133 Milano, Italy
[c] Dipartimento di Scienze Animali, Facoltà di Medicina Veterinaria, Università degli Studi di Udine, via San Mauro 2, 33010 Pagnacco (UD), Italy
[d] Department of Microbiology, Faculty of Agriculture, University of Zagreb, Svetosimunska, 25 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 13 May 2008
Received in revised form 15 July 2008
Accepted 17 July 2008
Available online 22 August 2008

Keywords:
Fermented sausages
Casings
Moulds
Ochratoxin A
Safety

ABSTRACT

The use of moulds as a seasoning for sausage can have both desirable and undesirable consequences. The desirable consequences are the creation of a successful product that appeals to consumers. The undesirable consequences are due to the growth of undesirable moulds that produce highly toxic secondary metabolites referred to as mycotoxins. The aim of the paper was to investigate the presence of moulds producing ochratoxin A (OTA) on the surface of sausages from northern Italy. A total of 757 mould strains were isolated from sausage casings. The most frequently identified species were *Penicillium nalgiovense*, *Penicillium oxalicum*, *Eurotium amstelodami*, *Penicillium olsonii*, *Penicillium chrysogenum*, *Penicillium verrucosum*, *Penicillium viridicatum*, and *Eupenicillium crustaceum*. *Aspergillus ochraceus* was detected in only one production lot. Approximately 45% of these samples were positive for the presence of OTA. On the casings of the investigated sausages, the lowest and highest OTA values were 3 and 18 μg/kg, respectively. The OTA concentration was reduced to below the limit of detection (LOD) by brushing and washing the sausages prior to sale. From these data it appears that the presence of OTA on the surface of sausage (on the casings) is not indicative of any health risk for human consumption of sausage, since OTA was not identified inside the dry meat.

1. Introduction

Several authors have studied the mycoflora of fermented sausages manufactured in Italy (Leistner and Eckardt, 1979; Dragoni and Cantoni, 1979; Dragoni et al., 1991; Grazia et al., 1986; Cantoni et al., 1982a,b, 2007; Mutti et al., 1988). The mycoflora is primarily heterogeneous, and although there are a variety of mycoflora, the predominant genus is *Penicillium*. In particular, *Penicillium nalgiovense*, and to a lesser extent *Penicillium chrysogenum* (Leistner, 1990), appear to be responsible for commercial covering and the seasoning of sausages. Both of these strains are used as starter cultures in sausage production (Leistner, 1986; Sunesen and Stahnke, 2003) and, as a consequence, they are spread on the sausage casings not only to improve but also to standardize the quality of sausage. For this reason the strains used during the fermenting process are carefully selected and added during the processing to ensure the safety of the final product (Lopez-Diaz et al., 2001; Comi et al., 2004a).

However, it is well known that other moulds can grow during sausage production. These moulds include non-mycotoxinogenic and mycotoxinogenic species, such as *Aspergillus ochraceus*, *Penicillium nordicum*, and *Penicillium verrucosum*, which are capable of producing ochratoxin A (OTA). These species were the primary toxinogenic species isolated from proteinaceous food such as sausages (Larsen et al., 2001), and potential health risk for those consumers that ingest cured pork meat.

OTA is a mycotoxin that represents the most important secondary metabolite produced by different moulds belonging to various species of *Penicillium* and *Aspergillus* (Leistner and Eckardt, 1981; Moss, 2000; Samson et al., 2004a,b).

P. verrucosum, *P. nordicum*, and *A. ochraceus* are all capable of producing OTA when they grow on the surfaces of foods, such as sausages, dry hams, and other meat and meat products during both ripening and storage (Leistner, 1984; Gareis and Scheuer, 2000; Spotti et al., 2001, 2002; Castella et al., 2002; Lund and Frisvad, 2003; Comi et al., 2004a,b; Matrella et al., 2006; Pietri et al., 2006; Cantoni et al., 1982a,b, 2007). In addition, *Aspergillus niger* is known to produce OTA on wine and grapes and to produce other toxic metabolites on food (Rojas et al., 1991; Abarca et al., 1994; Nunez et al., 1996; Serra et al., 2003; Samson et al., 2004a; Perrone et al., 2006),

* Corresponding author. Tel.: +39 0432 558129; fax: +39 0432 558130.
E-mail address: giuseppe.comi@uniud.it (G. Comi).

0740-0020/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2008.07.006

but whether it produces OTA on other foods is unknown. Other species of *Penicillium* and *Aspergillus*, such as *Aspergillus carbonarius* (Abarca et al., 2001), may also produce OTA, but they have yet to be found on meat products.

Recently, the issue of mould toxicity has attracted the attention of the food industry since the environmental conditions in the manufacturing rooms used for sausage production are very suitable for mould growth on the surfaces of products (Mizakovà et al., 2002; Comi et al., 2004a). This may explain the presence of mycotoxins inside the meat or on the sausage casing. The presence of OTA in foodstuffs is certainly undesirable and it has been classified by the International Agency for Research of Cancer (IARC) as a possible human carcinogen in Group "2B". Several studies have shown it to have teratogenic, neurotoxic, genotoxic, immunotoxic, and nephrotoxic properties (I.A.R.C., 1993; Moss, 2000; JEFCA, 2001; Luhe et al., 2003; Schaaf et al., 2002; Alvarez et al., 2004; Lioi et al., 2004). The highly nephrotoxic activity can produce both acute and chronic kidney lesions (I.A.R.C., 1993; Matrella et al., 2006). OTA has also been implicated as the cause of an endemic disease characterized by progressive renal fibrosis and urinary tract tumours in humans living in the Balkans (I.A.R.C., 1993; Matrella et al., 2006). Given that the legal limit for OTA in meat and meat products in Italy is 1 μg/kg (Circolare Ministero Sanità no. 10-09/06/1999) and that the presence of OTA in foods has been linked to health risks, the aim of the work was to investigate the presence of moulds and OTA on the surface of dry sausages produced traditionally and industrially.

2. Materials and methods

2.1. Sampling procedures

One hundred artisanal sausages and 60 industrial sausages were collected from different plants in northern Italy (Milano, Bergamo, Mantova and Cremona area). The sausages were classified into different types: Milano, Varzi, Brianza, Napoli, Ungherese, Cremonese, Bergamasco, and Mantovano types (Table 1). After ripening, the sausages were entirely covered in a light slime consisting of a variety of moulds, and the colour was predominantly white and a greenish-grey. The weight of these sausages was about 400 g each. The sausages were then washed and the moulds were brushed away before selling them to local and regional markets. There was no mould starter (*P. nalgiovense*) added during the production of sausages in artisanal plants, whereas *P. nalgiovense* was added to sausages produced in industrial plants.

For our analysis, sausages were collected prior to the last step of the production process, which entails washing and brushing the casings to remove the moulds. Mycological samples were subsequently taken from all of the sausages by swabbing the entire surface of each with a cotton swab wetted with sterile, physiological saline solution (0.7% NaCl, 0.05% Tween 80). The cotton swabs were then streaked onto malt extract agar plates (Oxoid, Milano, Italy) and incubated at 25 °C for 3–5 days. In cases where a sausage had a significant number of mould layers, samples were taken by directly inoculating the same agar plates with small fragments removed from the surface layer using a sterile needle.

Table 1
Type and number of sausages tested

Type of sausages	Number of artisanal tested sausages	Number of industrial tested sausages
Bergamasco	12	6
Brianza	10	10
Cremonese	12	6
Mantovano	12	6
Milano	20	10
Napoli	7	6
Ungherese	7	6
Varzi	20	10
Total	100	60

2.2. Mould identification by traditional methods

The colonies of the moulds grown on Malt Agar (Oxoid, Milano, Italy) were inoculated onto three different agars: Czapek Dox agar (Pitt, 1973); malt agar (Oxoid, Milano, Italy); and salt–malt agar pH 6.2, containing 5% malt extract (Oxoid, Milano, Italy), 5% NaCl, and 2% agar (Oxoid, Milano, Italy). The moulds were identified based on morphology and growth characteristics according to Ainsworth et al. (1973), Pitt (1987), Pitt and Hocking (1997), Samson and Pitt (2000), and Samson et al. (2004b).

2.3. Mould identification by molecular methods

A molecular method was used in order to eliminate any uncertainty in mould identification based on the aforementioned traditional methods, and to identify strains that could not be identified using traditional methods. One hundred isolates were tested using both methods and were subsequently compared to determine accuracy.

2.3.1. DNA extraction from pure culture

DNA extraction was performed using the DNeasy Plant Kits (Qiagen, Milan, Italy) starting from about 500 mg of mycelium. DNA was re-suspended in 50 μl of sterile water and used for PCR amplification.

2.3.2. PCR protocol and mould identification

DNA amplification was performed using primers NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and NL4 (GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman and Robnett, 1997). The reaction mixture (100 μl) was prepared with 2 μl of template DNA (50 ng), 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.2 mM dNTPs, 1.25 U *Taq* polymerase (Applied Biosystems, Milan, Italy), and 0.2 μM of each primer. PCR was performed in a PTC-220 DNA Engine Dyad MJ Research Thermal Cycler (Celbio, Milan, Italy). The protocol included 30 cycles of denaturation at 95 °C for 60 s, annealing at 48 °C for 45 s, and extension at 72 °C for 60 s. The cycle was preceded by an initial denaturation step at 95 °C for 5 min and followed by a final extension step at 72 °C for 7 min. Five microliters of each PCR reaction were analyzed by electrophoresis in a 0.5X TBE-agarose gel.

After purification using QIAquick PCR Purification Kit (QIAGEN, Milan, Italy), amplicons (680 bp) were sent to a commercial facility for sequencing (MWG Biotech, Edersberg, Germany). Sequences were aligned in GenBank using the Blast program (Altschul et al., 1997) in order to determine the closest known relatives of the moulds based on the partial 26S rDNA sequence obtained.

2.4. Mycotoxin detection

The presence of OTA was investigated in samples taken from both the sausage casing and from the sausage at a depth of 0.5 cm underneath the sausage casing and surface.

2.4.1. Preparation of sausage samples casings

Casings were completely removed from the sausages using a sterile lancet and pliers under a vertical laminar flow hood. The casings were homogenized and 10 g or 20 g of the homogenate was used for the tests.

2.4.2. Preparation of sausage samples meat

Samples (100 g) of meat were taken from a depth of 0.5 cm under the sausage surface and homogenized. Aliquots of 10 g or 20 g were used for the analysis.

2.4.3. ELISA and HPLC analysis

Hydrochloridic acid (1 M, 17.5 ml) was added to 10 g of the homogenized casing or meat, and the mixture was extracted with 20 ml of dichloroethane. The extract was then assayed for OTA using an ELISA kit (Ridascreen Ochratoxin A; R-Biopharm, Italy) (Matrella et al., 2006).

Samples that were both positive and negative for the presence of OTA using the ELISA test were analyzed by high performance liquid chromatography (HPLC) following the procedure in Matrella et al. (2006). Briefly, the extraction technique involved homogenizing 20 g of each sample of casing or meat with 6 ml of 1 M phosphoric acid for 5 min. An aliquot (2.5 g) of the homogenate was extracted with 5 ml of ethyl acetate and centrifuged for 5 min at 2500*g*. The organic phase was removed and the residue was re-extracted. Both of the organic phases were combined, reduced to 3 ml, and back-extracted with 3 ml of 0.5 M $NaHCO_3$, pH 8.4. The aqueous layer was acidified to pH 2.5 with 7 M H_3PO_4 and briefly sonicated to remove the CO_2 that had formed. OTA was extracted with 3 ml of ethyl acetate and the organic phase was evaporated to dryness under a N_2 stream, reconstituted in 150 μl of the mobile phase, and an aliquot (20 μl) was injected into the HPLC. The chromatographic conditions were exactly those of Matrella et al. (2006).

Initial values of OTA concentrations were determined using ELISA and then the HPLC method was used to confirm the results. The ELISA method, which is a semi-quantitative technique, is an invaluable tool for rapidly screening samples. However, the ELISA method tends to slightly underestimate the OTA concentration with respect to HPLC (Matrella et al., 2006), so the ELISA results were compared to the HPLC measurements using statistical analysis.

2.5. Reduction of OTA from sausages

Three artisanal and three industrial samples for each type of sausage were randomly collected, with each set of three samples coming from the same production lots (48 total samples). The samples were brushed and washed before analyzing the OTA content. The methods of sampling and of detection used were the same as described above.

2.6. OTA production "in vitro"

Five strains of each of the different mould strains (*Penicillium viridicatum*, *P. chrysogenum*, *P. verrucosum*, *P. nordicum*, *Penicillium olsonii*, *Penicillium oxalicum*, *Aspergillus versicolor*, *Aspergillus glaucus* and *Eurotium amstelodami*) isolated from sausages were tested *in vitro* in order to evaluate their potential ability to produce OTA. The single strain of *A. ochraceus* was also isolated and analyzed. The isolates were inoculated onto Czapek Dox agar plates (CYA; Pitt, 1987) that contained 5 g/l yeast extract (Oxoid, Italy) and were incubated at 25 °C for 10 days in the dark. The OTA production on agar was qualitatively detected using thin layer chromatography according to the methods described by Lopez-Diaz et al. (2001) and Paterson and Bridge (1994). Briefly, after the mould growth on an agar culture was homogenized in a chloroform/methanol mixture (2:1, v/v; 50 ml/plate), the resulting sample was treated and filtered with anhydrous sodium sulphate. The filtrate was partially evaporated to dryness in a rotary evaporator under an N_2 stream. Subsequently, 20 μl of the dried extracts were dissolved in 1 ml chloroform and were spotted onto silica gel plates (Merck, No. 5554) together with a standard solution of OTA (Sigma, St. Louis, MO, USA). The solvent and the method used to identify and visualize OTA were described by Paterson and Bridge (1994).

2.7. Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance. The means were separated by Tukey's honest significant difference test using a StatGraphics software package from Statistical Graphics (Rockville, Maryland, USA).

3. Results and discussion

A total of 757 strains were isolated from the casing of different sausages produced in a variety of industrial and artisanal production facilities of northern Italy (Table 2). The most frequently detected species were *P. nalgiovense*, *P. oxalicum*, *E. amstelodami*, *P. olsonii*, *P. chrysogenum*, *P. verrucosum*, *P. viridicatum*, *Eupenicillium crustaceum*, *P. nordicum*, and *A. ochraceus*. The last one is the main *Aspergillus* species thought to be responsible for OTA production. However, this strain of *Aspergillus* was isolated from only one production lot. Additional species were found but at a significantly lower frequency.

As expected, we detected *P. nalgiovense* and *P. chrysogenum*, which have been identified in meat products by several investigators (Dragoni and Cantoni, 1979; Grazia et al., 1986; Leistner, 1986, 1990). Both of these species are considered the typical starter cultures for sausage production. In Europe, these species are inoculated on the surface of sausages because they are thought to significantly affect processing (Leistner, 1984; Grazia et al., 1986; Lucke, 1997). Specifically, these species are thought to affect sausage aroma, texture, and appearance; resistance to abrasion, and height and density of mycelium growth on the surface. Nevertheless, whether or not *P. chrysogenum* is suitable as a starter culture remains uncertain (Sunesen and Stahnke, 2003).

In Italy, *P. nalgiovense* is the main starter culture used for sausage production, which explains why it was detected on the surface of all the industrial sausages analyzed. In comparison, this species was isolated from only 40% of the artisanal sausages; artisanal production facilities do not use mould starters. This allows for spontaneously colonising flora to predominate and thus the composition of species isolated from casings varies at each production site, even though *Penicillium* spp. are the most revalent mycoflora present at the end of sausage processing (Dragoni and Cantoni, 1979; Grazia et al., 1986; Andersen, 1995;). The existence of

Table 2
Moulds isolated from the surface of sausages using sterile cotton swabs

Species of moulds	Number of moulds isolated	Number of artisanal sausages infected	Number of industrial sausages infected
Alternaria alternata	3	3	–
Aspergillus ochraceus	1	1	–
Aspergillus penicillioides	4	4	–
Aspergillus versicolor	45	30	15
Eupenicillium crustaceum	60	25	35
Eurotium amstelodami	95	50	45
Eurotium rubrum	7	–	7
Penicillium chrysogenum	80	30	50
Penicillium commune	12	12	–
Penicillium expansum	10	5	5
Penicillium griseofulvum	2	–	2
Penicillium hirsutum	5	–	5
Penicillium nalgiovense	120	60	60
Penicillium nordicum	35	18	17
Penicillium olsonii	95	60	35
Penicillium oxalicum	100	60	40
Penicillium verrucosum	63	35	28
Penicillium viridicatum	15	10	5
Scopulariopsis flava	5	5	–
Total sausages infected	160	100	60

spontaneous mycoflora, which sometimes predominates on starter cultures, is demonstrated by the presence of a vast number of other mould species isolated from the analyzed sausages.

The relative prevalence of mould strains growing on casings depends on the type of sausage, the area of production, and the technological parameters of production. These parameters include water activity (A_w), humidity, temperature and the relative humidity of the drying and ripening steps (Dragoni and Cantoni, 1979; Grazia et al., 1986; Mutti et al., 1988; Lucke, 1997; Mizakovà et al., 2002; Sunesen and Stahnke, 2003; Comi et al., 2004a,b; Cantoni et al., 2007). If good manufacturing practices are not followed, or mistakes occur in the control of the relative humidity and temperature of the drying and ripening rooms, a significant growth of slime consisting of various moulds is often observed. These moulds can include *Penicillium* spp., *Aspergillus* spp., *Scopulariopsis* spp. and *Alternaria* spp., and all of these species can produce mycotoxins, as previously demonstrated by Cantoni et al. (1982a).

Using a combination of traditional and molecular methods, we unambiguously determined the identity of all (757) the isolated strains. In particular, 45 strains were identified only by molecular methods, including *P. olsonii* (25), *Penicillium hirsutum* (4), *Penicillium expansum* (5), and *E. crustaceum* (7). Interestingly, the identification of 100 strains using both methods gave the same results. These species included *P. verrucosum*, *P. nordicum*, *P. oxalicum*, *P. olsonii*, *P. chrysogenum*, *P. viridicatum*, *E. amstelodami*, *Alteraria alternata*, *A. ochraceus*, *Aspergillus penicilloides*, and *A. versicolor*. Thus, the traditional methods employed here are equally effective and specific as the molecular methods, although the latter are superior since they allow unambiguous identification. In addition, the traditional methods can be difficult to implement because they require culturing the mould strains, performing microscopic analysis, and testing for mycotoxin production (Pitt, 1987; Larsen et al., 2001). Furthermore, analyzing secondary metabolites is suggested for distinguishing between *P. viridicatum* and *P. verrucosum* (Pitt, 1987).

In the present work 160 samples were examined. These samples were collected directly from the processing plant at the end of the ripening period, prior to being prepared for sale. This means that the slime of moulds on these samples had not been washed from the sausage casing. The OTA concentration in these samples was determined on the casings and inside the meat at a depth of 0.5 cm below the casing surface.

The ELISA test was used as an initial screen to determine the presence of OTA, based on a limit of detection (LOD) of 0.1 μg/kg. All ELISA samples, whether positive or negative for OTA, were then analyzed by the HPLC method of Matrella et al. (2006). OTA was detected by the ELISA method in approximately 45% of the samples, i.e. 37 of 100 artisanal sausages and 35 of 60 industrial sausages. Conversely, the OTA concentration in 88 of 160 (55%) samples was lower than the ELISA LOD (<0.1 μg/kg). The percentage of industrial sausages with OTA levels greater than the LOD was higher than the percentage of artisanal sausages.

The results suggest that the use of a starter culture based upon *P. nalgiovense* does not limit the growth of mycotoxinogenic moulds on the surface of industrial sausages. This may be because of the processing conditions' variability and absence of quality checks throughout the production cycle. Thus the uncontrolled growth of moulds may cause an increase in OTA on casings, as previously demonstrated by Cantoni et al. (1982a). To be sure, it is not unusual in Italy to dry several lots of sausage casings with different ages in the same drying room, which makes it difficult to maintain appropriate conditions such as relative humidity and temperature. This variability in growth conditions often produces abnormal growth of both mycotoxinogenic and non-mycotoxinogenic housekeeping moulds on the surface of sausages, even when starter cultures are used.

Although the ELISA method has been commercialized for the detection of OTA in cereals and serum, Frohlich et al. (1997) and Matrella et al. (2006) have argued that ELISA tends to slightly underestimate OTA levels compared to HPLC. Thus, in the present study, we tested all of our sausage samples using HPLC (Fig. 1) after they had been assayed by ELISA. For all of our samples, the ELISA data were determined not to be statistically different from the data obtained by the HPLC method ($p > 0.05$). On the surface (casings) of the investigated sausages, the lowest and highest OTA values were 3 and 18 μg/kg, respectively (Table 3), and the means varied from 4.5 to 8.0 μg/kg. All of the OTA-positive samples exceeded the OTA limit suggested in the Circolare Ministero Sanità (no. 10-09/06/1999, Italian Ministry of Health). No significant differences in OTA concentration were observed among the different types of sausages, nor were there significant differences between artisanal and industrial sausages ($p > 0.05$).

Interestingly, no OTA was observed inside the dry meat for any of the sausage samples tested. It is possible that OTA was present, but at a concentration below the LOD of 0.1 μg/kg. This result contrasts with that obtained in previous Italian studies. Spotti et al. (2002) and Pietri et al. (2006) detected low levels of OTA in excess of 1.0 μg/kg inside meat samples. Chiavaro et al. (2002) and Zannotti et al. (2001) obtained similar results in dry cured ham. The fact that we failed to detect OTA inside the sausages in the present study may indicate that the raw meat did not contain OTA, and OTA did not diffuse through the casing into the meat.

Moulds are capable of producing mycotoxins under suitable conditions (Ostry, 2001), and the production of mycotoxins in meat and meat products can be fostered by the presence of oxygen, temperatures between 4 and 40 °C, pH values between 2.5 and 8.0 (with the optimum value between 5.0 and 8.0), a minimum A_w of 0.80, and a maximum salt concentration of 14% (Moss, 2000). In fact, mycotoxinogenic moulds produce mycotoxins on the sausage casings but not inside the meat since the lack of oxygen inside the meat precludes their growth (Moss, 2000). Therefore the presence of mycotoxins in the meat of sausages is probably due to the use of contaminated raw meat or a casing that permits diffusion from outside, as demonstrated by Cantoni et al. (1982a), who detected diffusion of OTA from *A. ochraceus* on the surface of sausages into the interior of the meat.

In the present work, the percentage of mycotoxinogenic moulds, specifically of *P. verrucosum* and *P. nordicum*, appears to explain the high concentration of OTA (>1 μg/kg) detected on the surface of the sausages. *P. verrucosum* was isolated in 63 of 72 OTA-positive sausages, and *P. nordicum* was isolated in 35 of 72 OTA-positive sausages. In some OTA-positive sausages, both *P. verrucosum* and *P. nordicum* were found, whereas *P. nordicum* was the only OTA producer in nine OTA-positive sausages. Only 98 (13%) of 752 of the strains identified are recognized as possible producers of OTA. Moreover several other possible OTA-producing strains

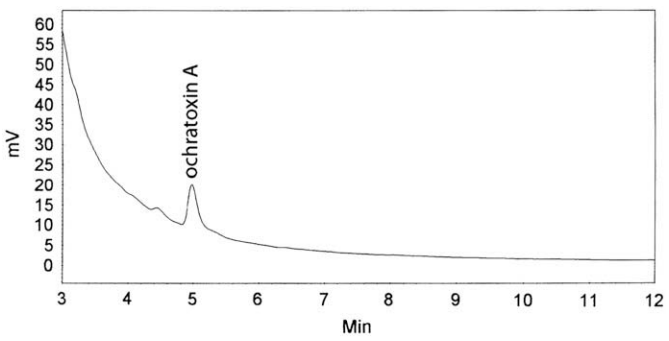

Fig. 1. Chromatogram of natural OTA contaminated samples.

Table 3
OTA levels in casings of various types of sausages produced in northern Italy

Type of sausages	Artisanal				Industrial				Range
	No.	IP	Mean	SD	No.	IP	Mean	SD	μg/kg
Milano	20	5	4.9	3.5	10	6	4.5	2.5	3–12
Varzi	20	7	6.0	1.5	10	5	5.6	3.0	5–10
Brianza	10	5	6.0	1.0	10	6	6.0	0.5	3–9
Napoli	7	3	7.0	1.0	6	4	7.5	0.5	4–13
Ungherese	7	3	6.0	2.5	6	4	5.0	1.5	3–18
Cremonese	12	5	5.8	0.5	6	4	5.5	0.5	3–10
Bergamasco	12	5	5.8	3.0	6	3	8.0	1.5	5–12
Mantovano	12	4	7.5	2.5	6	3	6.0	0.5	4–15
Total	100	37 (37%)			60	35 (58.3%)			

No.: number of samples; IP: incidence of positives: >LOD (=0.1 μg/kg); mean/SD: μg/kg.
Percentage of OTA positives (); percentage of total positives: 45%; HPLC method.

(Czerwiecki, 2001), belonging to *Penicillium cyclopium*, *P. viridicatum*, *P. chrysogenum*, *P. verrucosum*, *P. nordicum*, *A. versicolor*, *A. ochraceus*, *A. glaucus*, and *E. amstelodami* were either not detected here or they were not the dominant mycoflora in the sausages tested. Various authors have already observed this anomaly in previous studies of the presence of OTA in dry cured hams and cereals (Spotti et al., 2001; Czerwiecki et al., 2002; Torelli et al., 2006). Nevertheless, we did identify the following strains and screened them for OTA production: *P. oxalicum*, *P. olsonii*, *P. chrysogenum*, *P. viridicatum*, and *E. amstelodami*. Our data show that these strains did not synthesize OTA *in vitro*; in contrast, *P. verrucosum* and *P. nordicum* did. Thus these strains must be considered responsible for the presence of OTA on the sausages in this study. These findings are consistent with those of Larsen et al. (2001) and Pitt (1987), both of which demonstrated that *P. verrucosum* and *P. nordicum* are the two primary *Penicillium* species responsible for OTA production.

OTA concentrations lower than the LOD were detected on the casings of 48 sausages that had been treated by brushing and washing to remove the mould. In Italy, consumers prefer to buy sausages without mould on the casing, so the producers are obliged to eliminate them by a variety of methods. In particular, it is common to spread rice flour on the surface of the ripened sausages after eliminating the mould layer by brushing, washing, or air pressure. According to our data, we suggest a method in which the sausage casing is brushed, then washed in order to reduce OTA and eliminate the potential risk to consumers' health.

4. Conclusions

Our work confirms that OTA is present in meat products, adding this class of foods to those in which it has already been detected: cereals, cereal products, coffee, beer, grape juice, dehydrated grapes, wine, cocoa products, nuts, spices, and animal feeds. In our study, OTA was found only on the sausage casings; no OTA levels above the LOD of 0.1 μg/kg were detected in the interior of the sausages (0.5 cm below the surface). This finding suggests that indirect transmission of OTA from animals exposed to contaminated feed to pork products occurs rarely, as reported by Pietri et al. (2006).

The only possible explanation for the high OTA levels detected in the present study is the growth of mycotoxigenic mould directly on the sausage casings. In addition, it appears that OTA is not present in the 0.5 cm outer layer of the sausage meat. Mycotoxigenic moulds originate from the air and the dehydrating and ripening rooms of the processing plants. These moulds can be present in greater amounts than the levels of moulds typical of meat products, or the moulds in starter cultures. The growth of these abundant "contaminating" moulds depends strictly on the technological process used. However the best methods to limit the presence and the growth of toxigenic moulds on the surface of dry sausages are to control the temperature and relative humidity during the sausage production, as well as the careful selection of protective mould starter cultures (Leistner, 1986, 1990; Comi et al., 2004b).

In conclusion it appears that the presence of OTA on the surface of sausage constitutes a health risk when moulds are not removed from the casings. Given that OTA was not identified in the dry meat, removal of moulds from the casing may limit or eliminate this risk entirely. Nevertheless we suggest the control of spices and raw meat, given that both can be contaminated by OTA (Gareis and Scheuer, 2000).

References

Abarca, M.L., Accensi, F., Bragulat, M.R., Cabanes, F.J., 2001. Current importance of ochratoxin A-producing *Aspergillus* spp. J. Food Prot. 64, 903–906.
Abarca, M.L., Bragulat, M.R., Castellà, G., Cabanes, F.J., 1994. Ochratoxin A production by strain of *Aspergillus niger* var. niger. Appl. Eniviron. Microbiol. 60 (7), 2650–2652.
Ainsworth, G.C., Sparrow, F.K., Sussman, A.S., 1973. A taxonomic review with keys: Ascomycetes and Fungi Imperfecti. In: Fungi, vol. IV. Academic Press, New York.
Altschul, S.F., Madden, T.L., Shaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acid Res. 25, 3389–3402.
Alvarez, L., Gil, A.G., Ezpeleta, O., Garcia Jalòn, J.A., Lòpez de Cerain, A., 2004. Immunotoxic effects of ochratoxin A in vistar rats after oral administration. Food Chem. Toxicol. 42, 825–834.
Andersen, S.J., 1995. Compositional changes in surface mycoflora during ripening of naturally fermented sausages. J. Food Prot. 58, 426–429.
Cantoni, C., Rossetti, R., Comi, G., Damieno, E., 1982a. Tossicità di insaccati crudi stagionati contaminati da *Aspergillus ochraceus*. Ind. Aliment. XXI (settembre), 630–634.
Cantoni, C., Rossetti, R., Dragoni, I., 1982b. Isolamento e determinazione di ocratossina A da insaccati crudi stagionati. Ind. Aliment. XXI (ottobre), 668–669.
Cantoni, C., Comi, G., Chiesa, L., Iacumin, L., 2007. Muffe e Ocratossina A negli insaccati crudi stagionati. Industrie Aliment. XLVI (gennaio), 1–4.
Castella, G., Larsen, T.O., Cabanes, J., Schmidt, H., Alberesi, A., Niessen, L., Farber, P., Geisen, R., 2002. Molecular characterization of ochratoxin A producing strains of the genus *Penicillium*. Syst. Appl. Microbiol. 25, 74–83.
Chiavaro, E., Lepiani, A., Colla, F., Bettoni, P., Pari, E., Spotti, E., 2002. Ochratoxin A determination in ham by immunoaffinity clean-up and a quick fluorimetric method. Food Add. Contam. 19, 575–579.
Comi, G., Orlic, S., Redzepovic, S., 2004a. Bacterial starter cultures for meat fermentation. In: Kniewald, Z. (Ed.), Current Studies of Biotechnology. Food, vol. III. Croatian Society of Biotechnology and Medicinska naklada, pp. 87–92. ISBN: 953-176-209-0.
Comi, G., Orlic, S., Redzepovic, S., Urso, R., Iacumin, L., 2004b. Moulds isolated from Istrian dried ham at the pre-ripening and ripening level. Int. J. Food Microbiol. 96, 29–34.
Czerwiecki, L., 2001. Ochratoxin A and other mycotoxins in Polish cereals and foods. Mycotoxin Res. 17, 125–128.
Czerwiecki, L., Czajkowska, D., Witkowska-Gwiazdowska, A., 2002. On ochratoxin A and fungal flora in Polish cereals from conventional and ecological farms. Part 1: occurrence of ochratoxin A and fungi in cereals in 1997. Food Add. Contam. 19, 470–477.
Dragoni, I., Cantoni, C., 1979. Le muffe negli insaccati crudi stagionati. Ind. Aliment. XVIII (aprile), 281–284.
Dragoni, I., Cantoni, C., Papa, A., 1991. Surface mycoflora of dry sausages in Carnia. Ind. Aliment. XXX, 842–844.
Frohlich, A.A., Marquardt, R.R., Clarke, J.R., 1997. Enzymatic and immunological approaches for the quantitation and confirmation of ochratoxin A in swine kidneys. J. Food Prot. 60, 172–176.
Gareis, M., Scheuer, R., 2000. Ochratoxin A in meat and meat products. Archiv fur Lebensmitt. Hyg. 51, 102–104.
Grazia, L., Romano, P., Bagni, A., Reggiani, D., Guglielmi, G., 1986. The role of moulds in the ripening process of salami. Food Microbiol. 3, 19–25.
I.A.R.C., 1993. Ochratoxin A. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. In: IARC Monograph on the evolution of carcinogenic risks to humans, vol. 56. International Agency for Research on Cancer, Geneva, pp. 489–521.
JEFCA, 2001. Ochratoxin A. First Draft 47 series.
Kurtzman, C.P., Robnett, C.J., 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. 35, 1216–1223.
Larsen, T.O., Svendsen, A., Smedsgaard, J., 2001. Biochemical characterization of ochratoxin A-producing strains of the genus *Penicillium*. Appl. Environ. Microbiol. 67 (8), 3630–3635.
Leistner, L., Eckardt, C., 1979. Workommen toxinogener *Penicilien* bei Flischerzeugnissen. Fleischwirt 59, 1892–1896.
Leistner, L., Eckardt, C., 1981. Schimmelpilze und Mycotoxine in Fleisch und Fleischerzeugnissen (Moulds and mycotoxins in meat and meat products).

In: Reii, J. (Ed.), Mykotoxine in Lebensmitteln. Gustav Fisher Verlag, Stuttgart, pp. 297–341.
Leistner, L., 1984. Toxigenic penicillia occurring in feeds and foods: a review. Food Technol. Australia 36, 404–413.
Leistner, L., 1986. Mold-ripened foods. Fleischwirt 66, 1385–1388.
Leistner, L., 1990. Mold-fermented foods: recent developments. Food Biotechnol. 4, 433–441.
Lopez-Diaz, T.M., Santos, J.A., Garcia-Lopez, M.L., Otero, A., 2001. Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of *Penicillium* isolates. Int. J. Food Microbiol. 68, 69–74.
Lioi, M.B., Santoro, A., Barbieri, R., Salsano, S., Ursini, M.V., 2004. Ochratoxin A and zearalenone: a comparative study on genotoxic effects and cell death induced in bovine lymphocytes. Mutat. Res. 557, 19–27.
Lucke, F.K., 1997. Fermented sausages. In: Wood, B.J.B. (Ed.), Microbiology of Fermented Foods. Blakie Academic and Professional, London, pp. 441–483.
Luhe, A., Hildebrand, H., Bach, U., 2003. A new approach to studying ochratoxin A (OTA)-induced nephrotoxicity; expression profiling in vivo and in vitro employing cDNA microarrays. Toxicol. Sci. 73 (2), 315–328.
Lund, F., Frisvad, J.C., 2003. *Penicillium verucosum* in wheat and barley indicates presence of ochratoxin A. J. Appl. Microbiol. 95, 1117–1123.
Matrella, R., Monaci, L., Milillo, M.A., Palmisano, F., Tantillo, M.G., 2006. Ochratoxin A determination in paired kidneys and muscle samples from swines slaughtered in southern Italy. Food Control 17, 114–117.
Mizakovà, A., Pipovà, M., Turek, P., 2002. The occurrence of moulds in fermented raw meat products. Czech J. Food Sci. 3, 89–94.
Moss, M.O., 2000. Toxigenic fungi and mycotoxins. In: Lund, B.M., Baird-Parker, T.C., Gould, G.W. (Eds.), The Microbiological Safety and Quality of Food, vol. II. Springer Science, New York, USA, pp. 1490–1517.
Mutti, P., Previdi, M.P., Quintavalla, S., Spotti, E., 1988. Toxinogenicity of mould strains isolated from salami as function of culture medium. Ind. Cons. 63, 142–145.
Nunez, F., Rodrìguez, M.M., Bermùdez, M.E., Còrdoba, J.J., Asensio, M.A., 1996. Composition and toxigenic potential of the mpould population on dry-cured Iberian ham. Int. J. Food Microbiol. 32, 185–197.
Ostry, V., 2001. Vyskyt plisni v mase a masnych vyrobcich (I). Vliv na zdravi clovéka. Maso 12, 20–24.
Paterson, R.R.M., Bridge, P.D., 1994. Biochemical techniques for filamentous fungi. IMI Technical Handbook, No. 1. CaB International, Surrey, UK.
Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and Amplified Fragment Length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72 (1), 680–685.
Pietri, A., Bertuzzi, T., Gualla, A., Piva, G., 2006. Occurrence of ochratoxin A in raw ham muscles and in pork products from northern Italy. Int. J. Food Sci. 1, 1–8.
Pitt, J.I., 1973. An appraisal of identification methods for *Penicillium* species: novel taxonomic criteria based on temperature and water relations. Mycologia 65, 1135–1157.
Pitt, J.I., 1987. *Penicillium viridicatum*, *Penicillium verrucosum*, and production of ochratoxin A. Appl. Environ. Microbiol. 53 (2), 266–269.
Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage. Blackie Academic & Professional, London.
Rojas, F.J., Jodral, M., Gosalvez, F., Pozo, R., 1991. Mycoflora and toxigenic *Aspergillus flavus* in Spanish dry-cured ham. Int. J. Food Microbiol. 13, 249–256.
Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M., Frisvad, J.C., 2004a. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. Stud. Mycol. 50, 45–61.
Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O., 2004b. Introduction to Food and Airborne Fungi, seventh ed. Centraalbureau Voor Schimmelcultures, Wageningen, The Netherlands.
Samson, R.A., Pitt, J.I., 2000. Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Harwood Academic Publisher, Amsterdam.
Schaaf, G.J., Nijmeier, S.M., Maas, R.F.M., Roestenberg, P., De Groene, E.M., Fink-Gremmels, J., 2002. The role of oxidative stress in ochratoxin A-mediated toxicity in proximal tubular cells. Biochim. Biophys. Acta 1588, 149–158.
Serra, R., Ambrunhosa, Kozakiewicz, Z., Venacio, A., 2003. Black Aspergillus species as ochratoxin producers in Portuguese wine grapes. Int. J. Food Microbiol. 88, 63–68.
Spotti, E., Chiavaro, E., Bottazzi, R., Del Soldato, L., 2002. Monitoraggio di ocratossina A in carne suina fresca. Ind. Cons. 77, 3–13.
Spotti, E., Chiavaro, E., Lepiani, A., Colla, F., 2001. Contaminazione da muffe e da ocratossina A in prosciutti stagionati e in fase di stagionatura. Ind. Cons. 76, 341–354.
Sunesen, L.O., Stahnke, L.H., 2003. Mould starter cultures for dry sausages-selection, application and effects. Meat Sci. 65, 935–948.
Torelli, E., Firrao, G., Locci, R., Gobbi, E., 2006. Ochratoxin A-producing strains of Penicillium spp. isolated from grapes used for the production of "passito" wines. Int. J. Food Microbiol. 106, 307–312.
Zannotti, M., Malagutti, L., Sciaraffa, F., Corti, M., 2001. Indagine preliminare sul contenuto di Ocratossina in salumi provenienti dalla grande distribuzione. In: Atti del 36° Simposio Internazionale di Zootecnia, 27 aprile 200l, p. 78.