Effect of *Penicillium nordicum* contamination rates on ochratoxin A accumulation in dry-cured salami.

Massimo Ferrara, Donato Magistà, Vincenzo Lippolis, Salvatore Cervellieri, Antoniaella Susca and Giancarlo Perrone*

Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Bari, Italy

*Corresponding author: *Massimo Ferrara*
e-mail: massimo.ferrara@ispa.cnr.it
Phone: +39 0805929376 Fax: +39 0805929374

Donato Magistà: donato.magista@ispa.cnr.it
Vincenzo Lippolis: vincenzo.lippolis@ispa.cnr.it
Salvatore Cervellieri: salvatore.cervellieri@ispa.cnr.it
Antoniaella Susca: antonella.susca@ispa.cnr.it
Giancarlo Perrone: giancarlo.perrone@ispa.cnr.it

Abstract

Fungal starter, such as *Penicillium nalgiovense*, are commonly used to inoculate sausages before seasoning process. However, *P. nordicum*, a well-known ochratoxin A (OTA) producer frequently isolated from seasoning rooms, could colonize~~d~~ the casing surface during the early stage of production. The relationship between OTA accumulation and simultaneous inoculation of *P. nalgiovense* and *P. nordicum* at different rates was evaluated. After 14 days of seasoning, the persistence of *P. nordicum* was assessed by LAMP assay revealing its capability to colonize and grow on salami surface at all the contamination rates. At the end of seasoning, OTA was accumulated both in mycelium and dry-cured meat when *P. nordicum* contamination rate ranged from 25% to 100% of inoculum, while no OTA was detected in dry-cured meat at 2.5% and 0.25%. Results demonstrated that contamination of fungal starter by *P. nordicum* could represent a serious concern during salami production and therefore represents an important critical point to be monitored.

Keywords

Penicillium nordicum; *Penicillium nalgiovense*; dry-cured meat; ochratoxin A; LAMP.

1. Introduction

The environmental conditions in the ~~manufacturing~~ drying rooms for sausages production are very suitable for mold growth on the surface of meat products. Fungal development on the surface of dry-cured meat products has an important role, in terms of quality, especially during the seasoning period of both industrially and artisanal products.

Although the surface mycoflora is very heterogeneous, the predominant genus is *Penicillium*. In particular, two atoxigenic species, *Penicillium nalgiovense*, and to a lesser extent *Penicillium chrysogenum* (Leistner, 1990), appear to be responsible for commercial covering and seasoning of sausages (Iacumin, Chiesa, Boscolo, Manzano, Cantoni, Orlic, & Comi, 2009). Moreover, the atoxigenic new species *P. salamii* has been recently described as very well adapted to this environment and frequently isolated from salami surface (Perrone, Samson, Frisvad, Susca, Gunde-Cimerman, Epifani, & Houbraken, 2015). However, the environmental conditions ~~in the~~ of manufacturing rooms ~~used~~ for salami production are suitable also for the growth ~~on the product surfaces~~ of undesirable molds on the surface of meat products (Comi, Orlic, Redzepovic, Urso, & Iacumin, 2004; Mižáková, Pipovà, & Turek, 2002) and thus the colonization by toxigenic fungal species could not be excluded.

The presence of mycotoxins in meat products or sausage casing is certainly undesirable, since they are secondary metabolites with relevant toxics effects on consumer health. It has been demonstrated that the presence of ochratoxin A (OTA) producing species, including *P. verrucosum* and *P. nordicum*, is the most serious concern in dry-fermented sausages (Battilani, Pietri, Giorni, Formenti, Bertuzzi, Toscani, Virgili, & Kozakiewicz, 2007; Iacumin et al., 2009). In particular, *P. nordicum* is a widely distributed contaminant of sodium chloride and protein rich food (such as salami and cured ham). The presence of OTA in dry-cured meat products ~~can~~ could also be due to indirect contamination of meat coming from carryover of animals exposed to naturally contaminated feed (Bertuzzi, Gualla, Morlacchini, & Pietri, 2013; Dall'Asta, Galaverna, Bertuzzi, Moseriti, Pietri, Dossena, & Marchelli, 2010).

OTA is a ~~potent~~ mycotoxin, with nephrotoxic, nephrocarcinogenic, teratogenic, neurotoxic and immunotoxic activities (EFSA, 2006; Marin-Kuan, Cavin, Delatour, & Schilter, 2008; Van Egmond, 2002). Moreover, it is involved in porcine and chicken nephropathy (Stoev, Dutton, Njobeh, Mosonik, & Steenkamp, 2010) and it is suspected to be one of the ~~the most important~~ etiological factors in human Balkan endemic nephropathy, as in the occurrence of tumors of urogenital tract (Pfohl-Leszkowicz, Petkova-Bocharova, Chernozemsky, & Castegnaro, 2002). OTA has been classified as possible human carcinogen Group 2B by the International Agency for Research of Cancer (IARC, 1993) and the Commission of European Communities fixed maximum admissible levels in several foodstuffs (Commission of the European Communities, 2006 and 2010). Although maximum admissible levels for OTA in meat and derived products are not yet established by European Commission, the Italian Ministry of Health has recommended a maximum value of 1 µg/kg in pork meat and derived products (Ministero della Sanità, 1999).

In order to counteract the colonization of salami surface from uncontrolled and heterogeneous fungal population, ~~and in particular by toxigenic species, controlled~~ fungal starter are used to inoculate sausages at the beginning of seasoning process. Commonly *P. nalgiovense* is used as starter culture to standardize and improve the quality of dry-cured sausages production (Sunesen & Stahnke, 2003). Its application is reported to exert a protective effect that avoids the colonization of dry-fermented sausages by undesirable fungal population, and in particular by toxigenic fungal species (Bernáldez, Córdoba, Rodríguez, Cordero, Polo, & Rodríguez, 2013). However, few data are available about accumulation of OTA in salami in relation ~~with~~ to different contamination rates of *P. nordicum* and on the protective effect of fungal starter against its colonization of sausages surface. In this regards, the lack of information is mainly due to inappropriateness of inoculating toxigenic species directly in the meat plants and thus the abovementioned aspects should be first investigated in controlled conditions. In the present work, the accumulation of OTA in salami

inoculated with different fungal starter mixtures of *P. nalgiovense* and *P. nordicum* was evaluated at a small scale seasoning process. Moreover, in order to evaluate the persistence of ochratoxigenic species ~~in~~ on salami during the seasoning process, a rapid and specific loop-mediated isothermal amplification (LAMP) assay has been applied for the specific detection of *P. nordicum* on salami surface.

2. Material and Methods

2.1 Fungal strain

The isolates *P. nordicum* ITEM 9634 and *P. nalgiovense* ITEM 15292 (Agri-Food Microbial Culture Collection of the Institute of Sciences of Food Production, CNR, Bari, Italy) were used for inoculation of sausages. ~~The f~~Fungal ~~cultures~~ strains were routinely cultured on PDA medium (potato extract 4 g/L, glucose 20 g/L, agar 15 g/L) at 25 °C for 5-7 days and stored as a spore suspension in 15% glycerol at -80° C.

2.2 Inocula preparation

Fungal inocula were prepared in 1 liter flasks containing 80 g of rice, soaked with distilled water to 30% w/v, incubated for one night at room temperature and sterilized at 121° C for 30 min. *P. nordicum* and *P. nalgiovense* were inoculated in different flasks by using a conidial suspension (10^5 conidia/mL) obtained by scraping conidia from colonies of the respective *Penicillium* species grown on PDA plates at 25 °C for 7 days. Inoculated flasks were incubated in the dark at 25 °C for 10 days. Conidia were recovered by washing the mycelium with a sterile solution of 0.05% (v/v) Tween 80 and filtering the suspension with sterile Miracloth paper (Calbiochem, San Diego, CA) to eliminate mycelium debris. The concentration of conidia was estimated by "Thoma" chamber and was adjusted to a final concentration of 10^7 conidia/mL for both *Penicillium* species. Different fungal inocula were prepared by mixing different amounts of *P. nalgiovense* and *P. nordicum* conidia as indicated in Table 1. Final conidia concentration for each inoculum was fixed at 10^6 conidia/mL.

2.3 Small scale seasoning process of salami

Fresh pork sausages, provided by Dodaro S.p.A., were manufactured according to the traditional formulation of sweet Calabrian sausage: 69.5% lean pork meat, 17.4% pork back fat, 8.8% bell pepper extract, 2.2 % sodium chloride, 0.7% microbial starter~~;~~ , 0.5% paprika, 0.4% dextrose, 0.2% sodium ascorbate, 0.2% fennel, 0.05% black pepper and 0.05% white pepper. Sausages were stuffed into a natural casing to obtain sausages of about 200 g each, linked together in chain of 5 pieces.

Sausages were stored at 4°C before being inoculated by immersion in the different fungal inocula. A total of 30 sausages, were inoculated and arranged in a STG 701 Glass Seasoning Cabinet (Everlasting S.r.l., Italy). The seasoning protocol consisted in a six phases ripening process of 30 days (Table 2). The seasoning experiment was performed twice on different batches of sausages.

2.4 *Penicillium nordicum* detection by LAMP assay

The presence of *P. nordicum* on the surface of inoculated sausages was evaluated after 14 days of seasoning by LAMP assay. A loopful of mycelium and spores was scraped from three different points of sausages surface, resuspended separately in 100 µl of distilled sterile water and incubated at 95°C for 5 min. After thermal treatment, samples were cooled on ice and centrifuged at 16000 g at 4°C for 5 min to separate cell debris. Then, 10 µl of crude DNA extract was directly used as template for LAMP assay (Ferrara, Perrone, Gallo, Epifani, Visconti & Susca, 2015). LAMP reactions were performed in triplicates for each extract.

2.5 Ochratoxin A determination in mycelium and dry-cured sausages

Ochratoxin A determination was carried out after 30 days of seasoning on both dry-cured meat and mycelium developed on sausages surface. In particular, the mycelium was collected, from each sausage, by scraping the surface of casing. Then, sausages were brushed and washed to remove any mycelium debris and then cut in small pieces, without removing the casing, for the subsequent OTA determination. OTA was extracted from mycelium with a mixture of methanol:acetonitrile:water (30:30:40; v/v/v) by blending for 30 seconds with a Sorvall Omnimixer (Sorvall Instruments, Norwalk, CN, USA). The ratio mycelium/extraction solvent mixture was 1 g/12 mL. After extraction the sample was filtered through a filter paper (Whatman N. 4) and the mycelium was discarded. The filtrate was diluted with an aqueous solution of $NaHCO_3$ (5% w/v; containing PEG 1% w/v) in a ratio 1:6 (v/v) and filtered through a glass microfiber filter (Whatman GF/A). Ten milliliters of the diluted filtered extract (equivalent to 0.139 g of mycelium) were passed through the OchraTest™ immunoaffinity column (VICAM, A Waters Business, Milford, MA, USA) at a flow rate of about 1 drop/s, followed by 5 mL of a washing solution (NaCl 2.5% w/v; $NaHCO_3$ 0.5% w/v) and 5 mL of water (flow rate of about 1-2 drop/s). OTA was then eluted with 2 mL of methanol and collected in a 4- mL silanized amber glass vial.

OTA determination in dry-cured meat samples was performed according to the method described by Chiavaro, Lepiani, Colla, Bettoni, Pari, and Spotti (2002), with minor modifications. In particular, after homogenization of whole sausages, samples were extracted with a mixture 70:30 (v/v) of methanol-1% sodium bicarbonate by blending at high speed for 3 min with a Steril Mixer

12 blender (International PBI, Milan, Italy). The ratio of dry-cured meat/extraction solvent mixture was 1:4 ~~g/4 mL~~(w/v). After filtration through filter paper (Whatman N. 4), 10 mL of filtrate was diluted with PBS-0.01% Tween 20 (PBS, sodium phosphate 10 mM, 0.85 % of NaCl, 0.01% Tween 20, pH = 7.4) in a ratio 1:5 (v/v) and then filtered through a glass microfiber filter (Whatman GF/A). Ten milliliters of filtered diluted extract (equivalent to 0.5 g of sample) were passed through the OchraTest™ immunoaffinity column (VICAM, A Waters Business, Milford, MA, USA) at a flow rate of about 1 drop/s. The immunoaffinity column was washed with 10 mL PBS-0.01% Tween 20 and 10 mL distilled water at a flow rate of 1–2 drops/s. OTA was eluted with 1.5 mL methanol and collected in a 4- mL silanized vial.

All eluted extracts were evaporated under a stream of air at ca. 50 °C, and the dried residue was reconstituted with 250 µL of a mixture acetonitrile/water/acetic acid (99:99:2, v/v/v). An aliquot of the solution (50 µL) was injected into the chromatographic apparatus. HPLC analyses were carried out using an Agilent 1100 Series chromatographic system (Agilent Technologies, Palo Alto, CA, USA) equipped with a fluorometric detector (model 363, λex= 333 nm, λem=460 nm). The analytical column was a Zorbax SB-C18 (5 µm, 4.6×150 mm; Agilent Technologies), preceded by a 4 x 3.00 mm SecurityGuard™ Cartridges (Phenomenex, Torrance, CA, USA). The flow rate of the mobile phase (acetonitrile/water/acetic acid, 99:99:2; v/v/v) was 1 mL/min (OTA retention time t = 6.5 min). The detection limits were 1 µg/kg and 0.2 µg/kg for mycelium and dry-cured meat samples, respectively.

3. Results and Discussion

The production process of dry-cured salami commonly required the external inoculation of fresh pork sausages with a fungal starter preparation, even though the use of controlled fungal starter is not so diffused, particularly among small manufacturers for production of artisanal salami. In Southern Italy, fungal inoculation of sausages is commonly originated from the natural "house flora" of seasoning rooms or retrieved from finished batches of dry-cured salami. In the former practice, the inoculation by natural "house flora" could not guarantee a rapid, homogeneous and controlled colonization of salami surfaces and therefore they are potentially much more exposed to the colonization of undesirable fungi that could compromise the quality and the safety of the final products. Instead, in the latter inoculation practice, inoculum retrieved from already finished salami (Alley, Cours, & Demeyer, 1992; Leroy, Verluyten, & Vuyst, 2006) might be most likely contaminated by conidia of undesirable and/or toxin-producing molds belonging from sporulating colonies. In both cases, undesirable contamination of fungal starter by toxigenic fungi could be

amplified during the seasoning process and represents an important critical issue for salami production.

Thus, in order to estimate the contribution of *P. nordicum* contamination to OTA accumulation in dry-cured products, in the present work the relationship between OTA accumulation and simultaneous inoculation of salami with *P. nordicum* and *P. nalgiovense* at different rates was ~~monitored~~ evaluated ~~at a small scale seasoning process~~. The direct inoculation of sausages with *P. nordicum* and their seasoning at a small scale contributed significantly to the novelty of the study due to the impossibility to inoculate OTA-producing strains directly in salami plants at industrial scale.

The inoculation of fresh pork sausages with inocula contaminated with *P. nordicum* at ~~the~~ different ~~inocula~~ rates simulated a possible ~~-~~condition that might occur during inoculation step of sausages before starting the seasoning process. Although *P. nordicum* and *P. nalgiovense* presented slight different eco-physiological traits (Samson & Frisvad, 2004), seasoning conditions used in this study ~~was~~ were effective to obtain a homogeneous development of fungal mycelium on the surface of salami with all the tested inocula. This showed ~~-~~that the inoculum concentration and the environmental conditions used for the small scale seasoning process were able to reproduce the seasoning process similarly to that commonly used in salami plants at industrial scale.

Since the isolation and identification of *P. nordicum* directly in salami plant is time consuming and requires sophisticated equipment and specific expertise~~, for the first time~~, a user friendly LAMP assay was used for the first time in order to speed-up the specific detection of *P. nordicum* on the surface of salami during a small scale seasoning process. The presence of *P. nordicum* was evaluated 14 days after inoculation~~for each different inocula~~ because of the presence on salami surface of a homogeneous mycelium, easy to be sampled. Based on LAMP results (Fig. 1a), *P. nordicum* was detected on the surface of sausages inoculated with inoculum B (0.25% *P. nordicum*), C (2.5% *P. nordicum*), D (25% *P. nordicum*), E (50% *P. nordicum*) and F (100% *P. nordicum*), revealing that *P. nordicum* when co-inoculated at 0.25% was able to colonize and grow on sausages surface even in the presence of *P. nalgiovense* at 99.75% in the inoculum. ~~This result~~Results confirmed the suitability of LAMP assay as non-destructive method for an early detection of ~~in detecting~~ the presence of *P. nordicum* DNA directly "on site" during the seasoning process, ~~also at low levels~~ offering indication on the possible up-coming OTA contamination in meat. However, as reported in previous studies for *P. verrucosum* (Bernáldez et al., 2013), the molecular detection of a toxigenic fungus did not necessarily imply the real presence of the toxin in the food matrix, being its biosynthesis closely related to secondary metabolism rather than primary one.

The presence of OTA was quantified both in dry-cured meat and mycelium grown on the surface of sausages after 30 days of seasoning process (Fig. 1b-1c). As expected, OTA was not detected in both mycelium and dry-cured meat for sausages inoculated with inoculum A. Otherwise~~In details~~, sausages inoculated with inoculum D, E and F showed OTA levels in mycelium of 110 µg/kg, 114 µg/kg and 83 µg/kg, while in dry-cured meat OTA levels were 1.8 µg/kg, 2.3 µg/kg and 2.0 µg/kg, respectively. These evidences indicated that the contamination of fungal inoculum by *P. nordicum* at a rate of 25 % could lead to OTA accumulation in meat above recommended maximum value in Italy. Likewise, higher contamination rates of *P. nordicum* determined an increase of OTA accumulation in meat at the end of seasoning process. Sausages inoculated with inoculum C showed OTA levels of about 5.1 µg/kg in the mycelium, while OTA was not detectable (LOD= 0.2 µg/kg) in dry-cured meat. Therefore, the detection of OTA in salami without eliminating the casing suggested that OTA did not accumulate significantly in the casing (data not shown), but probably spreads gradually in the meat in relation to OTA amount accumulated in the mycelium. Based on our results, the amount of OTA detected in meat was about 1 % of the total amount detected in the mycelium (as observed for inocula D, E and F) and therefore the presence of casing plays an important role as barrier to OTA migration in meat. ~~Moreover,~~ In the case of inoculum B, although LAMP assay revealed the presence of *P. nordicum* after 14 days, OTA contamination was not detected in both mycelium and dry-cured meat. ~~for sausages inoculated with inoculum B and inoculum A.~~

~~In the case of inoculum B,~~ Thus, in the present study the protective effect of *P. nalgiovense* ~~could be hypothesized~~, ~~as~~ already reported against *P. nordicum* and *P. verrucosum* on dry-cured sausages (Bernáldez et al., 2013; Comi, Chiesa, Panseri, Orlic, & Iacumin, 2013). ~~However, in the present study the protective effect of *P. nalgiovense*~~ was not observed if higher concentrations of *P. nordicum* conidia were used to co-inoculate salami surface ~~together with *P. nalgiovense*~~. In this regards, other study are need to deeper investigate the nature of their interaction and to clarify if the observed effects was related to the ability of *P. nalgiovense* strain to interfere with OTA production or mycelial growth of *P. nordicum*.

Conclusions

The contamination of fungal starters, such as *P. nalgiovense*, by ochratoxigenic fungi could represent a serious concern in the production of dry-cured sausages. The inoculation of sausages with an inoculum contaminated even with low conidia concentrations of *P. nordicum* could lead to the accumulation of valuable OTA levels at the end of seasoning process. Indeed, the application directly “on site” of a user friendly rapid, specific and sensible method, such as LAMP assay, may

be of great value in the monitoring or verification procedures to detect the presence of toxigenic molds in HACCP plans. ~~This~~Our evidence suggested that the analysis of growing mycelium on the surface of ripening salami for OTA content and *P. nordicum* presence using rapid detection tool, could represent a good practice to monitor a possible up-coming OTA accumulation throughout the seasoning process.

Therefore, when OTA-producing molds are detected on the surface of dry-cured meat during the seasoning process, corrective actions could be promptly adopt to avoid the consumption of dry-cured meat products contaminated by OTA.

Conflict of interest

There is no conflict of interest.

Acknowledgements

This work has been supported by the Italian Ministry of Education, University and Research, MIUR (P.O.N. "Ricerca & Competitività" 2007-2013), project no. PON01_01409 - SAFEMEAT "Process and product innovations aimed at increasing food safety and at diversifying pork-based products". We thanks Dodaro S.p.A and Mr. Franco Longo for providing fresh pork sausages and technical assistance for the small scale seasoning experiments.

References

Alley, G., Cours, D., & Demeyer, D. (1992). Effect of nitrate, nitrite and ascorbate on color and color stability of dry, fermented sausage prepared using back slopping. *Meat Science, 32,* 279–287.

Battilani, P., Pietri, A., Giorni, P., Formenti, S., Bertuzzi, T., Toscani, T., Virgili, R., & Kozakiewicz, Z. (2007). *Penicillium* populations in dry-cured ham manufacturing plants. *Journal of Food Protection, 70,* 975-980.

Bernáldez, V., Córdoba, J. J., Rodríguez, M., Cordero, M., Polo, L., & Rodríguez, A. (2013). Effect of *Penicillium nalgiovense* as protective culture in processing of dry-fermented sausage "salchichón". *Food Control, 32,* 69-76.

Bertuzzi, T., Gualla, A., Morlacchini, M., & Pietri, A. (2013). Direct and indirect contamination with ochratoxin A of ripened pork products. *Food Control, 34,* 79-83.

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 fluorometric method. *Food Additives & Contaminants, 19,* 575-581.

Comi, G., Orlic, S., Redzepovic, S., Urso, R., & Iacumin, L. (2004). Moulds isolated from Istrian dried ham at the pre-ripening and ripening level. *International Journal of Food Microbiology, 96,* 29–34.

Comi, G., Chiesa, L., Panseri, S., Orlic, S., & Iacumin, L. (2013). Evaluation of different methods to prevent *Penicillium nordicum* growth on and ochratoxin A production in country style sausages. *World Mycotoxin Journal, 6,* 411–418.

Commission of the European Communities. (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union, L 364,* 5e24.

Commission of the European Communities. (2010). Commission Regulation (EC) No 105/2010 of 5 February 2010 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. *Official Journal of the European Union, L 35,* 7-8.

Dall'Asta, C., Galaverna, G., Bertuzzi, T., Moseriti, A., Pietri, A., Dossena, A., & Marchelli, R. (2010). Occurrence of ochratoxin A in raw ham muscle, salami and dry-cured ham from pigs fed with contaminated diet. *Food Chemistry, 120,* 978–983.

EFSA. (2006). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food. *EFSA Journal, 365,* 1-56.

Ferrara, M., Perrone, G., Gallo, A., Epifani, F., Visconti, A., & Susca, A. (2015). Development of loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Penicillium nordicum* in dry-cured meat products. *International Journal of Food Microbiology, 202,* 42-47.

Iacumin, L., Chiesa, L., Boscolo, D., Manzano, M., Cantoni, C., Orlic, S., & Comi, G. (2009). Moulds and ochratoxin A on surfaces of artisanal and industrial dry sausages. *Food Microbiology, 26,* 65–70.

IARC, International Agency for Research on Cancer. (1993). *IARC monographs on the evaluation of the carcinogenic risk to humans,* 56 (pp. 449). Lyon, France: IARC Press.

Leistner, L. (1990). Mold-fermented foods: recent developments. *Food Biotechnology, 4,* 433–441.

Leroy, F., Verluyten, J., & Vuyst, L. D. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology, 106,* 270-285

Marin-Kuan, M., Cavin, C., Delatour, T., & Schilter, B. (2008). Ochratoxin A carcinogenicity involves a complex network of epigenetic mechanisms. *Toxicon, 52,* 195-202.

Ministero della Sanità (1999). Circolare 09/06/1999, n. 10. Gazzetta Ufficiale della Repubblica Italiana, 135, 11 giugno 1999.

Mižáková, A., Pipovà, M., & Turek, P. (2002). The occurrence of moulds in fermented raw meat products. *Czech Journal of Food Sciences, 3,* 89–94.

Perrone, G., Samson, R. A., Frisvad, J. C., Susca, A., Gunde-Cimerman, N., Epifani, F., & Houbraken, J. (2015). *Penicillium salamii*, a new species occurring during seasoning of dry-cured meat. *International Journal of Food Microbiology, 193,* 91–98.

Pfohl-Leszkowicz, A., Petkova-Bocharova, B., Chernozemsky, I. N., & Castegnaro, M. (2002). Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins. *Food Additives & Contaminants, 19,* 282-302.

Samson, R.A., & Frisvad, J.C. (2004). *Penicillium* subgenus *Penicillium*: new taxonomic schemes, mycotoxins and other extrolites. *Studies in Mycology, 49,* 1-157.

Stoev, S. D., Dutton, M. F., Njobeh, P. B., Mosonik, J. S., & Steenkamp, P. A. (2010). Mycotoxic nephropathy in Bulgarian pigs and chickens: complex aetiology and similarity to Balkan endemic nephropathy. *Food Additives & Contaminants, 27,* 72-88.

Sunesen, L. O., & Stahnke, L. H. (2003). Mould starter cultures for dry sausages-selection, application and effects. *Meat Science, 65,* 935–948.

Van Egmond, H. P. (2002). Mycotoxins: detection, reference materials and regulation. In Samson, R.A., Hoekstra, E.S., Frisvad, J.C., & Filtenborg, O. (Eds.), *Introduction to Food and Airborne Fungi* (pp. 332–338). Utrecht: Centraalbureau voor Schimmelcultures.

Figure captions

Figure 1: Comparison between *P. nordicum* persistence and OTA production. *In-tube* LAMP assay reactions on mycelium scraped after 14 days of seasoning (a); OTA accumulation after 30 days of seasoning in mycelium (b) and dry-cured meat (c). Capital letters indicate the different inocula used to inoculate fresh pork sausages: A) 100% *P. nalgiovense*; B) 99.75% *P. nalgiovense* + 0.25% *P. nordicum;* C) 97.5% *P. nalgiovense* + 2.5% *P. nordicum;* D) 75% *P. nalgiovense* + 25% *P. nordicum;* E) 50% *P. nalgiovense* + 50% *P. nordicum;* F) 100% *P. nordicum*. Bars indicate standard errors of three biological replicates.

Table 1

Table 1: Different inoculum combinations with increasing amount of *P. nordicum* conidia used for the seasoning experiments. For each combination the percentage and respective estimated conidia concentration of *P. nalgiovense* and *P. nordicum* are reported.

Inoculum ID	*P. nalgiovense*	*P. nordicum*
A	100% (10^6 conidia/mL)	-
B	99.75% ($9.975 \cdot 10^5$ conidia/mL)	0.25% ($2.5 \cdot 10^3$ conidia/mL)
C	97.5% ($9.75 \cdot 10^5$ conidia/mL)	2.5% ($2.5 \cdot 10^4$ conidia/mL)
D	75% ($7.5 \cdot 10^5$ conidia/mL)	25% ($2.5 \cdot 10^5$ conidia/mL)
E	50% ($5.0 \cdot 10^5$ conidia/mL)	50% ($5.0 \cdot 10^5$ conidia/mL)
F	-	100% (10^6 conidia/mL)

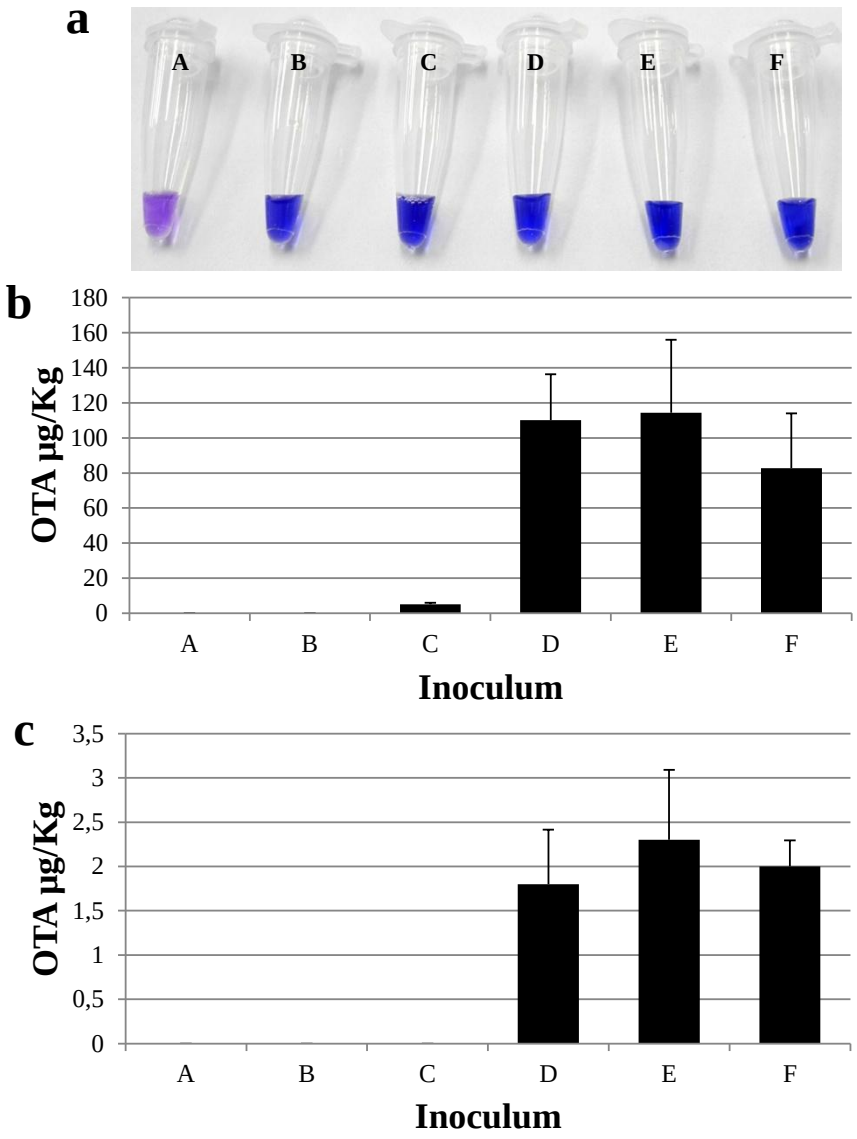
Table 2

Table 2: Protocol used for the seasoning process of sausages.

Parameters	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6
T (°C)	22	20	18	16	14	12
RH (%)	90	60	70	75	80	85
D (hour/day)	4 hours	1 day	2 days	2 days	2 days	23 days

T=temperature; RH=relative humidity; D=duration

Figure 1


a
A
B
C
D
E
F
b
OTA µg/Kg
180
160
140
120
100
80
60
40
20
0
A
B
C
D
E
F
Inoculum
c
OTA µg/Kg
3,5
3
2,5
2
1,5
1
0,5
0
A
B
C
D
E
F
Inoculum