Rapid prediction of ochratoxin A-producing strains of *Penicillium* on dry-cured meat by MOS-based electronic nose

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Abstract

The availability of rapid diagnostic methods for monitoring the ochratoxigenic species during the seasoning processes is crucial and constitutes a key stage in order to prevent the risk of ochratoxin A (OTA) contamination. A rapid, easy-to-perform and non-invasive method using an electronic nose (e-nose) based on metal oxide semiconductors (MOS) was developed to discriminate dry-cured meat samples in two classes based on the fungal contamination: class P (samples contaminated by OTA-producing *Penicillium* strains) and class NP (samples contaminated by OTA non-producing *Penicillium* strains). Two OTA-producing strains of *P. nordicum* and two OTA non-producing strains of *P. nalgiovense* and *P. salamii*, were tested. The feasibility of this approach was initially evaluated by e-nose analysis of 480 samples of both Yeast Extract Sucrose (YES) and meat-based agar media inoculated with the tested *Penicillium* strains and incubated up to 14 days. The high recognition percentages (higher than 82%) obtained by Discriminant Function Analysis (DFA), either in calibration and cross-validation (*leave-more-out* approach), for both YES and meat-based samples demonstrated the validity of the used approach. The e-nose method was subsequently developed and validated for the analysis of dry-cured meat samples. A total of 240 e-nose analyses were carried out using inoculated sausages, seasoned by a laboratory-scale process and sampled at 5, 7, 10 e 14 days. DFA provided calibration models that permitted to discriminate dry-cured meat samples after only 5 days of seasoning with mean recognition percentages in calibration and cross-validation of 98 and 88%, respectively. A further validation of the developed e-nose method was performed using 60 dry-cured meat samples produced by an industrial-scale seasoning process showing a total recognition percentage of 73%. The pattern of volatile compounds of dry-cured meat samples was identified and characterized by a developed HS-SPME/GC-MS method. Seven volatile compounds (2-methyl-1-butanol, octane, 1R-α-pinene, D-limonene, undecane, tetradecanal, 9-(Z)-octadecenoic acid methyl ester) allowed to discriminate between dry-cured meat samples of classes P and NP. These

results demonstrate that MOS-based electronic nose can be a useful tool for a rapid screening in preventing OTA contamination in the cured meat supply chain.

Keywords

Electronic nose, metal oxide sensors, rapid method, *Penicillum nordicum*, dry-cured meat.

1. Introduction

Dry-cured meat products, such as dry-fermented sausages, constitute one of the most representative traditional foods that are produced and consumed in different areas of the world. Their importance into the market is rapidly increasing and consumers strictly demand high quality and safe products (Asefa et al., 2010).

Fungal development on the surface of dry-cured meat products has an important role on their production, in terms of quality, especially during the seasoning period of both industrially and handmade products. The quality of raw materials, physical and biochemical factors, manufacturing practices and the hygienic quality of the production environment determine the types of fungi growing on dry-cured meat products (Mizakova et al., 2002; Samson et al., 2004). A composite mycobiota was observed on the surface of these products with *Aspergillus*, *Eurotium,* and *Penicillium* as the most frequently isolated genera (Battilani et al., 2007; Comi et al., 2004, López-Díaz et al., 2001; Papagianni et al., 2007; Sonjak et al., 2011; Soresen et al., 2008). Among these genera *Penicillium* species, such as *P. nalgiovense*, *P. chrysogenum*, *P. olsonii*, *P. solitum* , were predominant mainly due to their use for the improvement of organoleptic characteristics of dry-cured meat and for preventing the growth of pathogenic, toxigenic or spoilage fungi (Samson et al., 2010; Sonjak et al., 2011; Soresen et al., 2008). At this regard, a new species described as *P. salamii* has been recently isolated and characterized on sausages during seasoning processes in the south of Italy (Perrone et al., 2015).

Although the fungal contamination contributes to improvement of the quality, some uncontrolled fungal development may also occur on these products leading either undesiderable alteration and synthesis of mycotoxins, such as ochratoxin A (OTA). OTA is a strong nephrotoxic agent and it has been shown to be teratogenic, mutagenic, hepatotoxic and immunosuppressive to animal species (Barlow et al., 2008; EFSA, 2006; Pfohl-Leszkowicz and Manderville, 2007). The International Agency for Research on Cancer (IARC) has classified OTA as possible carcinogenic to humans (group 2B) (IARC, 1993).

Penicillium nordicum is the most important OTA-producing species frequently isolated from dry-cured meats (Bogs et al., 2006; Frisvad and Thrane, 2002; Samson et al., 2002). The presence of OTA in dry-cured meat products can be related to direct contamination with moulds or to indirect contamination of meat coming from carryover of animals exposed to naturally contaminated feed (Bertuzzi et al., 2013; Dall'Asta et al., 2010; Gareis, 1996). Therefore, it is crucial to prevent and monitor possible contamination of meat by OTA-producing species to avoid undesirable negative economic impact and a potential health hazard to consumers. Conventional approaches to fungal detection in food involve morphological identification or molecular detection (Bogs et al., 2006; Ferrara et al., 2015; Iacumin et al., 2009). Although, these techniques are highly specifics and reliable, they are time consuming, expensive and require specialist expertise.

For these reasons the development of rapid and easy-to-use methods for early detection of OTA-producing species during the seasoning process of meat is highly demanded in order to reduce OTA contamination risk in dry-cured meat products. Analysis of volatile compounds has been shown to be often used for early detection of food spoilage, fungal growth and also for distinguishing between toxigenic and non-toxigenic strains (Magan and Evans, 2000; Vinaxia et al., 2004). Since the volatile headspace of real matrix is complex it should be evaluated as a whole using electronic noses based on non-specific sensors. Moreover electronic nose methods represent convenient tools for their rapidity, simplicity and low costs. Electronic nose technology has been applied for discriminating mycotoxigenic and non mycotoxigenic strains, such as *Fusarium verticillioides*, *Aspergillus flavus*, *A. carbonarius*, *P. verrucosum* and *P. nordicum* (Cabañes et al., 2009; Falasconi et al., 2005; Keshri and Magan, 2000; Leggieri et al., 2011; Shagal et al., 2007). In particular, Leggieri et al. (2011) demonstrated the potential use of electronic nose analysis to analyse qualitative volatile patterns produced by *P. nordicum* and discriminate between OTA-producing and non-producing strains on a ham-based medium.

The aim of this work was to develop and validate an electronic nose-based method for early detection of OTA-producing and non-producing strains of *Penicillium* during the seasoning process of dry-cured meat products. In particular, an electronic nose based on metal oxide semiconductors (MOS) was initially trained by using YES and meat-based agar media inoculated with OTA-producing and non-producing strains of *Penicillium*. Multivariate statistical analysis was used to discriminate the inoculated samples on the basis of the pattern of volatile organic compounds. This approach was subsequently used to develop and validate an e-nose method for rapid prediction of OTA-producing and non-producing strains of *Penicillium* in dry-cured meat samples produced both at laboratory- and industrial-scale. In addition a HS-SPME/GC-MS method was developed to characterize the pattern of volatile compounds specifically related with the presence of OTA-producing and non-producing strains of *Penicillium* on the surface of dry-cured meat samples.

2. Materials and Methods

2.1 Reagents and apparatus

Potato dextrose agar (PDA) was purchased from Biolife (Milan, Italy), Yeast extract and agar technical were purchased from Oxoid (Basingstoke, United Kingdom), sucrose was purchased from Carlo Erba Reagents (Milan, Italy).

Acetonitrile (HPLC grade), methanol (HPLC grade), sodium chloride (ACS grade), polyethylene glycol (PEG 8000), TWEEN® 20, phosphate-buffered saline (PBS) and acetic acid were purchased from Sigma-Aldrich (Milan, Italy). Sodium hydrogen carbonate ($NaHCO_3$, ACS grade) were purchased from Mallinckrodt Baker (Milan, Italy). OchraTest™ immunoaffinity columns were purchased from VICAM, a Waters Business (Milford, MA, USA). Filter paper (No. 4) and glass microfibre filters (GF/A) were obtained from Whatman

153 (Maidstone, U.K.). Ultrapure water was produced by a Millipore Milli-Q system (Millipore,
154 Bedford, MA, USA).
155 Ten milliliters headspace vials with crimp cap composed by a pierceable silicon/PTFE
156 septa were purchased from Varian Inc. (Turin, Italy). PTFE syringe filter with diameter of 25
157 mm and pore size of 0.20 µm were bought by Teknokroma (Barcelona, Spain).
158 Chromatographic air (80% N_2, 20% O_2) was obtained by Sapio s.r.l. (Bari, Italy).
159 The manual solid-phase microextraction (SPME) sampler holder and 50/30 µm
160 divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 85 µm polyacrilate (PA),
161 75 µm carboxen/polydimethylsiloxane (CAR/PDMS) and 65 µm
162 polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers were purchased from Supelco
163 (Bellafonte, PA, USA). Trans-3-hexen-1-ol (≥98%) were obtained from Aldrich Chemical Co.
164 (Milwaukee, WI).
165
166 *2.2 Fungal strains*
167 The fungal species tested for this study were selected on the base of their frequency
168 of occurrence on the surface of sausages during seasoning processes in the production of
169 salami. In particular, two non-OTA producing strains of *Penicillium nalgiovense* ITEM 15292
170 and *P. salamii* ITEM 15302, and two OTA-producing *P. nordicum* strains ITEM 9634 and
171 ITEM 13080, respectively, were used in the study. *P. nalgiovense* and *P. salamii* were
172 previously isolated from a salami plant in Calabria (Italy) and identified by morphological and
173 molecular analysis (Perrone et al., 2015). All *Penicillium* strains are from the Agri-Food
174 Toxigenic Fungi Culture Collection (www.ispa.cnr.it/Collection/) of the Institute of Sciences
175 of Food Production (ISPA-CNR, Bari, IT). Working cultures were maintained on PDA
176 medium at 25°C for 5-7 days and stored as conidial suspension in 15% v/v glycerol at -20°C.
177
178 *2.3 Inoculation, incubation and seasoning processes*

179 *2.3.1 Laboratory-scale trials*

180 *In vitro* assays (fungal inoculation and incubation of media) were performed on YES
181 agar (Yeast extract 20 g/L, sucrose 150 g/L, agar 20 g/L) and meat agar plates (fresh pork
182 meat from sausages powdered in liquid nitrogen 30 g/L, agar 20 g/L), previously sterilized
183 by autoclaving at 121°C for 15 min. The conidial suspension was obtained by scraping the
184 surface of PDA plates inoculated with the respective *Penicillium* strain after 7 days of
185 incubation at 25°C. The recovered conidia were resuspended in 10 mL of sterilized distilled
186 water. Conidia concentration was determined by using a “Thoma” chamber.

187 A total of fifteen replicates of YES agar and meat agar plates were inoculated with 0.1
188 mL of a conidial suspension of 10^4 conidia/mL, respectively. Not inoculated plates of both
189 media were prepared and used as reference samples in the HS-SPME/GC-MS analysis.
190 Plates were incubated at 25°C in the dark and 2 g (about six agar plugs of 16 mm Ø) were
191 sampled from each inoculated and not inoculated replicates after 5, 7, 10, 14 days and were
192 sealed in 10 mL vials. All these procedures were carried out as duplicated experiments.

193 Fresh pork sweet sausages (about 200 grams each), were provided by a salami plant
194 in Calabria (Italy) and were prepared according to the manufacture’s formulation. For
195 sausages inoculum preparation, flasks containing 80 g of rice, soaked in distilled water to
196 30% w/v, were incubated over night at room temperature and then sterilized by autoclaving
197 at 121°C for 30 min. The flasks were inoculated with 5 mL of a conidial suspension (10^5
198 conidia/mL) of each fungal strain and incubated in the dark at 25 °C for 6 days. Conidia were
199 recovered by washing the mycelium with 500 mL of sterile distilled water and filtering the
200 suspension with sterile gauze. The conidial suspension was adjusted to 10^6 conidia/mL. The
201 sausages were inoculated with the respective *Penicillium* strains by dipping them in the
202 conidial suspension. For each *Penicillium* strain were inoculated 20 sausages. After
203 inoculation, before starting the seasoning process, three sausages were randomly sampled
204 from each batch to evaluate the fungal colonization on the external surface of the gut. The

sampled sausages were immersed in 200 mL of sterile water and an appropriate serial dilution was plated onto Dichloran Rose Bengal Chloramphenicol Agar (DRBC agar) and incubated at 25 °C in the dark for 48-72 h. Final concentrations were expressed as Colony Forming Unit (CFU)/cm^2.The inoculated sausages were seasoned at laboratory-scale in an Everlasting Seasoning cabinet (Everlasting S.r.l., Italy) for 14 days, following a standard seasoning protocol adapted to the duration of our trials, as indicated in Table 1.

Two different seasoning cabinets were used: one for the inoculum of the OTA-producing *Penicillium* strains, and the other for the *Penicillium* OTA non-producing strains. An aliquot (2 g) of inoculated sausages was sampled after 5, 7, 10, 14 seasoning days and was sealed in 10 mL vials. Samples in the sealed 10 mL vials were directly analyzed by e-nose and HS-SPME/GC-MS.

2.3.2 *Industrial-scale trials*

A set of sausages were inoculated with *P. nalgiovense* and *P. salamii* as described in chapter 2.3.2 and seasoned by an industrial-scale process in a salami plant (Calabria, Italy) according to the manufacture's protocols. In order to respect the internal safety protocol of salami plant, none sausages were inoculated with *P. nordicum* strains. Ten sausages were inoculated for each *Penicillium* strain. Fifteen aliquots (2 g) of inoculated sausages were sampled after 5, 7, 10, 14 seasoning days and sealed in 10 mL vials. Samples in the sealed 10 mL vials were directly analyzed by e-nose and HS-SPME/GC-MS.

2.4 Electronic nose analysis

An Artificial Olfactory System (AOS) ISE Nose 2000 (SoaTec S.r.l., Parma, Italy) was used, being formed of an array of 12 Figaro thick layer Metal Oxide Semiconductors (MOS). The electronic nose was equipped with a 16-position autosampler, a thermal unit and a humidity trap. The digital fingerprint $\Delta R/R_0$, where ΔR is the difference between R and R_0 (R

231 is the instant resistance and R_0 is the resistance at the beginning of the acquisition), was
232 recorded as function of time for each sensor. The analyses were carried out by maintaining
233 at a constant temperature of 40 °C the 10 mL headspace vials containing YES agar, meat
234 agar or dry-cured meat samples to generate the volatile components in the headspace and
235 injected in the sensors chamber under the following conditions: carrier gas, chromatographic
236 air (80% N_2, 20% O_2); gas flow, 300 mL/min; temperature, 40 °C; gas humidity, stabilized at
237 12 g/m^3; headspace generation time, 600 s; baseline acquisition time, 5 s; injection time, 15
238 s; data acquisition time, 180 s; time delay between samples, 180 s.
239 A total of 480 analyses was performed on both inoculated YES agar and meat agar
240 samples (60 inoculated plates at 4 incubation days, as duplicated experiments) originating
241 two different groups of data sets. These data sets were used in a preliminary study of
242 calibration and validation aimed to assess the feasibility of the electronic nose analysis in
243 discriminating OTA-producing and non-producing strains of *Penicillium*.
244 A set of 240 analyses was successively performed on dry-cured meat samples (60
245 inoculated sausages at 4 incubation days) seasoned at laboratory-scale and used for the
246 calibration and validation of the electronic nose-based method to discriminate samples
247 contaminated by OTA-producing and non-producing strains of *Penicillium* during seasoning
248 processes.
249 Sample replicates were analyzed by randomly changing the position into the
250 autosampler during the entire period of analysis. In each data set the $\Delta R/R_0$ traces belonging
251 to the most discriminating sensors and relevant features were chosen by the software
252 (ISENose OCS, revision 02A). All features were implemented in the discriminant function
253 analysis (DFA) for the classification of OTA-producing (class P) and non-producing (class
254 NP) strains of *Penicillium*. The pattern recognition technique chosen was the canonical DFA
255 coupled with a cross-validation process that generate a recognition percentage rate (R_r) per
256 each sample. The cross-validation was performed by *leave-more-out* procedure, excluding

257 10% of samples randomly selected by software from each entire data set. Ten iterations
258 were used in the cross-validation process and R_r were calculated and reported as mean
259 values.
260 An external validation was also performed by analyzing dry-cured meat samples (n =
261 60) obtained by industrial-scale processes using the developed method and calibration
262 models obtained for meat samples seasoned at laboratory-scale. Misclassification due to
263 data over-fitting was avoided by keeping the samples to variables ratio (f) higher than 3 in
264 all data treatments (Huberty and Olejnik, 2006; Massart et al., 1988).
265
266 *2.5 Ochratoxin A determination*
267 Ochratoxin A (OTA) was extracted from mycelium of YES and meat agar with a mixture
268 of methanol:acetonitrile:water (30:30:40; v/v/v) by blending for 30 seconds with a Sorvall
269 Omnimixer (Sorvall Instruments, Norwalk, CN, USA) at room temperature. The ratio
270 mycelium/extraction solvent mixture was 1 g/12 mL. After extraction the sample was filtered
271 through a filter paper (Whatman N. 4) and the mycelium was discarded. The filtrate was
272 diluted with an aqueous solution of $NaHCO_3$ (5% w/v; containing PEG 1% w/v) in a ratio 1:6
273 (v/v) and filtered through a glass microfiber filter. Ten milliliters of the diluted filtered extract
274 (equivalent to 0.139 g of mycelium) was passed through the immunoaffinity column at a flow
275 rate of about one drop per second, followed by 5 mL of a washing solution (NaCl 2.5% w/v;
276 $NaHCO_3$ 0.5% w/v) and 5 mL of water (flow rate of about one/two drops per second). OTA
277 was then eluted with 2 mL of methanol and collected in a 4-mL silanized amber glass vial.
278 The eluted extract was evaporated under a stream of air at ca. 50 °C, and the dried residue
279 was reconstituted with 250 µL of a mixture acetonitrile/water/acetic acid 99:99:2 (v/v/v). An
280 aliquot of the solution (50 µL) was injected into the chromatographic apparatus.
281 OTA determination in dry-cured meat samples was performed according to the method
282 described by Chiavaro et al. (2002).

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, λ_{ex}= 333 nm, λ_{em}=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 dry mycelium and dry-cured meat samples, respectively.

2.6 HS-SPME/GC-MS analysis

A total of 16 samples of meat agar and sausages inoculated with the tested *Penicillium* strains (ITEM 9634, ITEM 13080, ITEM 15292 and ITEM 15302) were analyzed by using an optimized HS-SPME/GC-MS method after 5, 7, 10 and 14 days of incubation or seasoning. Eight meat agar samples not inoculated were similarly analyzed and used as reference. All these analyses were performed as duplicated experiments. In particular, 2 g sample were placed in a 10 mL headspace vial and kept at temperature of 40 °C for 10 min, in a water bath, to generate the volatile components headspace. The extraction from the headspace was performed exposing a polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber at 40 °C for 30 min. After extraction, compounds were thermally desorbed in the injection port of the gas chromatograph at 250 °C for 5 min. The analyses were carried out by an Agilent 6890 Series GC system (Agilent Technologies, Palo Alto, CA, USA) with an Agilent 5973 Network Mass Selective Detector mass spectrometer using a DB-5MS (30 m x 0.25 mm i.d., 0.25 µm film thickness, Agilent) fused-silica capillary column. The injection port fitted with a 0.75 mm i.d. liner was maintained at 250 °C in splitless mode. The analyses were performed with programmed temperature: initial temperature 40 °C maintained for 5 min, from 40 to 150 °C at 3 °C/min, 150 to 280 °C at 25 °C/min, the final temperature being maintained for 5 min.

The helium flow rate was held constant at 1 mL/min. The transfer line, ion source and quadrupole temperatures were 280, 290 and 150 °C, respectively. Electron impact Ionization (EI+) mode with an electron energy of 70 eV was used. The mass spectrometer acquired data in full scan mode (scan range: 40-350 amu). The compounds were identified by comparison of experimental mass spectra with ones present in the Wiley 138 and NIST 02 libraries database using a match quality higher than 70. Quantification of compounds was performed by the method of internal standardization using *trans*-3-hexen-1-ol at a concentration of 3 µg/g. The amount of each identified compound was estimated by comparing the total ion current (TIC) peak area with *trans*-3-hexen-1-ol peak area and expressed as area units.

3. Results and discussion

3.1 Prediction of OTA-producing strains by e-nose

3.1.1 YES agar and meat agar media

Determination of OTA carried out by HPLC analysis on YES agar and meat agar samples inoculated with the four selected *Penicillium* strains after 14 incubation days showed the presence of OTA in plates inoculated with *P. nordicum* strains with contamination values ranging from 40 to 139000 µg/kg, whereas OTA was not detectable for all replicates inoculated with *P. nalgiovense* and *P. salamii*.

Inoculated YES and meat agar plates were classified in two established groups: class P, for samples inoculated with OTA-producing strains of *Penicillium* (ITEM 9634 and ITEM 13080); class NP, samples inoculated with non-producing strains of *Penicillium* (ITEM 15292 and ITEM 15302). Two groups of data sets were obtained from 480 analyses performed by using the optimized electronic nose method either on YES and meat agar sampled at 5, 7, 10 and 14 incubation days. Discriminant Function Analysis (DFA) was applied to signals,

335 $\Delta R/R_0$ as function of time, choosing the most discriminating sensor (6 traces out of 12
336 recorded) and features (n= 3). A samples to variables ratio (f) of 3.33 was obtained in all
337 data sets.

338 Table 2 shows recognition percentages and relevant mean values for the two classes
339 P and NP for YES agar and meat agar samples obtained in calibration at four incubation
340 times (5, 7, 10 and 14 days). Recognition percentages and mean values obtained for the
341 two classes in calibration ranged from 98 to 100% for both YES and meat agar samples at
342 the different incubation days.

343 Validation process were carried out with cross-validation approach using *leave-more-*
344 *out* procedure excluding 10% of samples from each data sets for both YES and meat agar
345 samples. Recognition percentages obtained in validation for the both P and NP classes
346 ranged from 82 to 98% with mean values in the range 89-95% for YES agar samples (Table
347 2). Similar results were obtained for meat agar samples with recognition percentages
348 ranging from 88 to 100% and mean values within the range 92-99%. These high recognition
349 percentages obtained either in calibration and validation for both YES and meat agar
350 samples demonstrated the applicability of the e-nose method to discriminate samples
351 belonging to two classes P and NP after only 5 incubation days. Therefore, the developed
352 e-nose method permitted a rapid prediction of the presence of *P. nordicum* contamination
353 and its discrimination from *Penicillium* non-producing species, i.e. *P. nalgiovense* and *P.*
354 *salamii*, nevertheless their similarity in terms of taxonomy and metabolic profiles.

355 These results obtained on inoculated media confirmed previous studies that showed
356 the feasibility of the e-nose analysis in discriminating samples contaminated by toxigenic
357 and non-toxigenic species, including *Aspergillus carbonarius* and *A. niger* on YES and WGJ
358 agar (Cabañes et al., 2009), *Fusarium verticillioides* and *F. proliferatum* on wheat meal agar
359 (Keshri and Magan, 2000), *Fusarium verticillioides* on a synthetic medium and maize grains
360 (Falasconi et al., 2005) and *P. nordicum* on ham-based medium (Leggieri et al., 2011).

Furthermore, all the acquired electronic nose signals were also used to evaluate the possibility to discriminate samples into four different classes of meat agar samples inoculated with the single strain of *P. nalgiovense* (ITEM 15292) and *P. salamii* (ITEM 15302) and two strains of *P. nordicum*. Inoculated samples were correctly classified for each strain with recognition percentages ranging from 80 to 100% in calibration and from 65 to 100% in validation. Figure 1 shows DFA analysis obtained for meat agar samples in the calibration process after 5 incubation days. These data demonstrated that the electronic nose could be also used to discriminated between meat agar samples contaminated by single strains of *Penicillium*.

3.1.2 Dry-cured meat

A set of dry-cured meat samples produced at laboratory-scale by seasoning sausages inoculated with different *Penicillium* strains (*P. nordicum*, ITEM 9634 and ITEM 13080; *P. nalgiovense*, ITEM 15292; *P. salamii*, ITEM 15302) was used for the development and validation of the electronic nose based method, following the same time-course analysis performed for YES and meat agar plates. After inoculation the fungal colonization on the external surface of the gut of dry-cured meat samples was estimated in 2,9 CFU/cm^2, 1,1 CFU/cm^2, 3,4 CFU/ cm^2 e 1,6 CFU/ cm^2 respectively for ITEM 9634, ITEM 13080, ITEM 15292 and ITEM 15302. No cross contamination on the sausages seasoned in the same cabinet and inoculated with different strains was observed. The HPLC analyses performed on dry-cured meat products confirmed the presence of OTA in samples inoculated with *P. nordicum* strains. The contaminations values ranging from 0.1 and 139 µg/kg whereas OTA was not detectable for all samples inoculated with *P. nalgiovense* and *P. salamii*.

Two groups of data sets were obtained from 240 analyses performed by using the optimized electronic nose method on dry-cured meat samples sampled at the different seasoning times, of 5, 7, 10 and 14 days. For these dry-cured meat samples DFA was

similarly applied to signals, $\Delta R/R_0$ as function of time, selecting the most discriminating sensor (4 traces out of 12 recorded) and features (n= 2). A samples to variables ratio (f) of 3.75 was obtained in all data sets.

Recognition percentages for the two established classes P and NP for dry-cured meat samples obtained in calibration at the four seasoning days (5, 7, 10 and 14 days) ranged from 87 to 100% (Table 3). Mean values of the measured recognition percentages were within 90 and 100%. Cross-validation performed with *leave-more-out* procedure, excluding 10% of samples from each data sets, showed recognition percentages for the both P and NP classes ranged from 73 to 97% with mean values in the range 83-95%.

These recognition percentages obtained either in calibration and validation for dry-cured meat samples demonstrated that the developed electronic nose method permits to distinguish the two classes P and NP after only 5 seasoning days. For this reason the developed e-nose method can be applied for early prediction of *Penicillium* OTA-producing strains during seasoning processes. However, the obtained calibration models should be further implemented by the use of additional samples contaminated by other potentially occurring, ochratoxigenic and non-ochratoxigenic, species of *Penicillium* in order to include their contribute in the patterns of volatile compounds detected by e-nose analysis.

An external validation of the developed electronic nose method was carried out analyzing 60 dry-cured meat samples produced by seasoning sausages at industrial-scale according to the manufacture's protocols. For all tested samples the analysis of fungal contamination after 5 seasoning days showed the presence of *P. nalgiovense* and *P. salamii* whereas OTA-producing strains was not detected on the surface of sausages. DFA showed that 44 out of 60 samples were correctly classified as class NP, corresponding to the 73% of the entire set of samples. Although the total recognition percentage was lower than those obtained by samples produced by laboratory-scale process it can be considered acceptable due to the predictable differences between samples obtained by laboratory- and industrial-

413 scale processes. A further improvement of the performances of the method in validation
414 process could be obtained by using in the calibration process dry-cured meat samples
415 produced by industrial-scale processes.
416
417 *3.2 Characterization of the pattern of volatile compounds by* HS-SPME/GC-MS
418 Volatile compounds were isolated from sausages during seasoning process by using
419 headspace solid-phase microextraction (HS-SPME) technique. A HS-SPME/GC-MS
420 method was *in-house* developed to characterize the pattern of volatile compounds related
421 to contamination by *Penicillium* strains in dry-cured meat samples. In particular, different
422 SPME fibers (DVB/CAR/PDMS, PA, CAR/PDMS, PDMS/DVB), extraction temperatures
423 (ranging from 20 to 80 °C), headspace equilibration times (ranging from 5 to 60 min) and
424 extraction times (ranging from 5 to 40 min) were tested for their efficacy in isolating volatile
425 compounds. Individual parameters were changed once at a time while keeping constant the
426 other parameters. The optimal experimental parameters, in terms of intensity and resolution
427 of peaks, for headspace analysis were DVB/PDMS fiber, extraction temperature 40 °C,
428 equilibration time of headspace 10 min and extraction time 30 min.
429 Volatile compounds of meat agar non-inoculated and inoculated with the tested
430 *Penicillium* strains were analysed under the optimized experimental conditions at 5, 7, 10 e
431 14 days of incubation. A total of 26 and 30 compounds were identified for non-inoculated
432 and inoculated meat agar samples, respectively, after 5 days of incubation. These volatile
433 compounds belonged to different chemical classes including saturated, unsaturated and
434 aromatic hydrocarbons, aldehydes, carboxylic acids, alcohols, ketones, esters and terpenic
435 compounds (Table 4). Volatile compounds have been shown to be species-specific and they
436 could serve as marker compounds for the selective detection of fungal species composition,
437 as well as to discriminate between toxigenic and non-toxigenic strains (Fiedler et al., 2001;
438 Sahgal et al., 2007) . However, Jeleñ and Grabarkiewicz-Szczęsna (2005) showed that

VOCs did not permit to differentiate OTA-producing and non-producing *Aspergillus ochraceus* strains, although the same authors affirmed that VOCs can be helpful in the discrimination of *Penicillium* strains.

For these reasons, time profiles of headspaces were analysed to identify a pattern of molecules that permit to discriminate between samples inoculated by OTA-producing (class P) and by non-producing strains of *Penicillium* (class NP). Although, 2-methyl-1-propanol, 1-octene-3-ol, styrene and 2-pentanone, which have been often correlated to the presence of *P. nalgiovense and P. nordicum* in meat-based products (Jacobsen and Hinrichsen, 1997; Karlshøj et al. 2007; Larsen and Frisvad, 1995; Sunesen et al., 2004) were found in all tested samples, they did not permit to differentiate samples belonging to classes NP and P. Nevertheless, a pattern of 10 volatile molecules, which permitted to discriminate between samples belonging to class NP and class P, was identify after only 5 days of incubation of inoculated agar samples. In particular, among the identified molecules, 2-butanone, 1R-α-pinene, 2-methyl-decane, 3-methyl-decane, D-limonene, undecane, tetradecanal and 9-(Z)-octadecenoic acid methyl ester were present at concentrations higher in samples of class NP than those of class P. Tetradecanal was the molecule showing the highest difference, of about four times, in the content ratios between the two classes. On the contrary, octane was found to be more abundant in samples of class P. In addition, 2-methyl-1-butanol was detected only for samples in class NP. Among the selected molecules, 2-butanone and 2-methyl-1-butanol have been already reported to be associated with *P. nalgiovense* and *P. nordicum* (Jacobsen and Hinrichsen, 1997; Karlshøj et al., 2007; Sunesen et al., 2004). Although the other selected molecules were related for the first time to the *Penicillium* strains object of this study, 1R-α-pinene, D-limonene, undecane and octane have been previously associated with other *Penicillium* species in meat-based products (Nilsson et al., 1996; Sunesson et al., 1995; Wihlborg et al., 2008).

The selected pattern was used to assess its applicability to dry-cured meat products during seasoning time for discriminating samples inoculated with OTA-producing and non-producing strains of *Penicillium*. Although, 2-butanone, 2-methyl-decane, 3-methyl-decane were not detected in the headspace of dry-cured meat samples the remaining seven volatile molecules was detected with contents permitting to differentiate the two groups of samples already after 5 days of seasoning process (Figure 2). In particular, 2-methyl-1-butanol, octane, 1R-α-pinene, D-limonene and undecane, showed higher contents in samples of class P than those of class NP. In particular, D-limonene and 1R-α-pinene were the molecules having the highest difference in content between dry-cured samples of class P and class NP with ratios of about 14 and 3, respectively. Tetradecanal was present at concentration higher in samples of class NP than those of class P. Finally, 9-(Z)-octadecenoic acid methyl ester was present only in samples of class P. The identification of a pattern of seven volatile compounds distinguishing OTA-producing and non-producing strains of *Penicillium* confirmed the relevance of this study as predictive tool for early prediction of fungal contamination during seasoning of sausages.

3. Conclusions

An electronic nose (e-nose) based on metal oxide sensors was successfully applied for rapid prediction of ochratoxin A-producing and non-producing *Penicillium* strains during the seasoning process of dry-cured meat products. The developed e-nose method was able to discriminate samples inoculated with ochratoxin A-producing and non-producing *Penicillium* strains after only 5 days of lab-scale seasoning of dry-cured meat with mean recognition percentages in calibration and validation of 98 and 88%, respectively. A total recognition percentage of 73% was obtained in the validation of the e-nose method using

dry-cured meat samples produced by industrial-scale seasoning process. In addition, a pattern of seven volatile compounds that permits to discriminate between dry-cured meat samples inoculated with ochratoxin A-producing and non-producing strains of *Penicillium* was identified and characterized by using a developed HS-SPME/GC-MS method.

This is the first study relevant to the application of a e-nose method to the early prediction of ochratoxigenic species during the seasoning of dry-cured meat products at laboratory- and industrial-scale.

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Highlights

- A MOS-based e-nose was applied to predict OTA-producing *Penicillium* strains
- E-nose discriminated OTA-producing strains with high recognition percentages (88-98%)
- Seven volatile compounds related to the presence of OTA-producing strains was characterized
- The first study of an e-nose applied to dry-cured meat at lab- and industrial-scale

Table 1. Protocol used in the laboratory-scale seasoning process of sausages.

Parameters	Phases					
	I	II	III	IV	V	VI
T (°C)[a]	22	20	18	16	14	12
RH (%)[b]	85	45	60	70	80	85
t_F (hours)[c]	4	24	48	48	48	164

[a]T = temperature

[b]RH = relative humidity

[c]t_F = time of phase

Table 2. Recognition percentages of the class P (samples inoculated with OTA-producer *Penicillium* strains) and class NP (samples inoculated with non-producer *Penicillium* strains), and mean values for YES agar and meat agar samples obtained in calibration and validation processes sampled at four incubation times (5, 7, 10 and 14 days).

Medium	Incubation time (days)	Calibration			Validation		
		P (%)	NP (%)	Mean (%)	P (%)	NP (%)	Mean (%)
YES agar	5	100	100	100	92	98	95
	7	100	98	99	95	95	95
	10	100	100	100	95	85	90
	14	100	100	100	82	96	89
Meat agar	5	100	100	100	98	93	96
	7	100	100	100	97	92	94
	10	100	100	100	98	100	99
	14	98	98	98	88	95	92

Table 3. Recognition percentages of the two classes P and NP and mean values for dry-cured meat samples obtained in calibration and validation processes at four seasoning times (5, 7, 10 and 14 days).

Seasoning time (days)	Calibration			Validation		
	P (%)	NP (%)	Mean (%)	P (%)	NP (%)	Mean (%)
5	100	97	98	83	93	88
7	87	93	90	73	93	83
10	97	93	95	93	97	95
14	100	100	100	83	83	83

732 Table 4. Volatile compounds (n=37) identified by HS-SPME/GC-MS analysis of meat agar
733 samples non-inoculated and inoculated with tested *Penicillium* strains (ITEM 9634,
734 ITEM13080, ITEM 15292 and ITEM 15302) after 5 days of incubation.

No	r_t[a]	Volatile Compound	Meat extract agar				
			Non-inoculated	*P.nalgiovense* (ITEM 15292)	*P.salamii* (ITEM 15302)	*P.nordicum* (ITEM 9634)	*P.nordicum* (ITEM 13080)
1	2.70	ethanol	+[b]	n.d.[c]	n.d.	n.d.	n.d.
2	3.30	2-butanone	+	+	+	n.d.	+
3	3.59	2-methyl 1-propanol	+	+	+	+	+
4	4.37	2-pentanone	+	+	n.d.	n.d.	+
5	5.23	1,1-diethoxy ethane	+	+	+	n.d.	+
6	5.51	3-methyl 1-butanol	+	n.d.	n.d.	n.d.	n.d.
7	5.63	2-methyl 1-butanol*	+	+	+	n.d.	n.d.
8	8.41	octane	n.d.	+	+	+	+
9	8.44	hexanal	+	n.d.	n.d.	n.d.	n.d.
10	9.85	1,3-octadiene isomer (I)	n.d.	+	+	+	+
11	9.93	1,3-octadiene isomer (II)	n.d.	+	+	+	+
12	13.52	styrene	+	+	+	+	+
13	14.27	heptanal	+	n.d.	n.d.	n.d.	n.d.
14	15.05	methoxy benzene	n.d.	n.d.	+	n.d.	n.d.
15	15.97	1R-α-pinene	+	+	+	+	+
16	17.36	Benzaldehyde	+	n.d.	n.d.	n.d.	n.d.
17	17.74	sabinene	+	n.d.	n.d.	n.d.	n.d.
18	18.15	1-octen-3-ol	+	+	+	+	+
19	19.15	Octanal	+	n.d.	n.d.	n.d.	n.d.
20	19.78	2-methyl 2-bornene	+	+	+	n.d.	+
21	20.04	p-cimene	+	+	+	n.d.	+
22	20.25	D-limonene	+	+	+	+	+
23	21.88	2-methyl decane	n.d.	+	+	+	+
24	22.16	3-methyl decane	n.d.	+	+	+	+
25	22.95	1,3,3-trimethyl 2-norbornanone	+	n.d.	n.d.	n.d.	+
26	23.54	undecane	+	+	+	+	+
27	26.56	1,3-dimethoxy benzene	n.d.	n.d.	+	n.d.	+
28	27.62	2-methyl isoborneol	+	+	+	n.d.	+
29	28.09	estragole	+	+	+	n.d.	+
30	32.72	tridecane	+	+	+	+	+
31	37.14	tetradecane	+	+	+	+	+
32	45.29	tetradecanoic acid	+	+	n.d.	+	+
33	45.66	tetradecanal	+	+	+	+	+
34	46.17	9-(Z)-hexadecenoic acid methyl ester	n.d.	+	+	+	+
35	47.11	9,12-(Z,Z)-octadecadienoic acid methyl ester	n.d.	+	+	+	+
36	47.13	9-(Z)-octadecenoic acid methyl ester	n.d.	+	+	+	+
37	47.25	octadecanoic acid methyl ester	n.d.	+	+	+	+

735 [a]r_t: retention time.
736 [b]+: detected compound
737 [c]n.d.: not detected
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741 Figure 1. Discriminant Function Analysis (DFA) for meat agar samples obtained in the
742 calibration process after 5 incubation days for the classification of single species of
743 Penicillium.

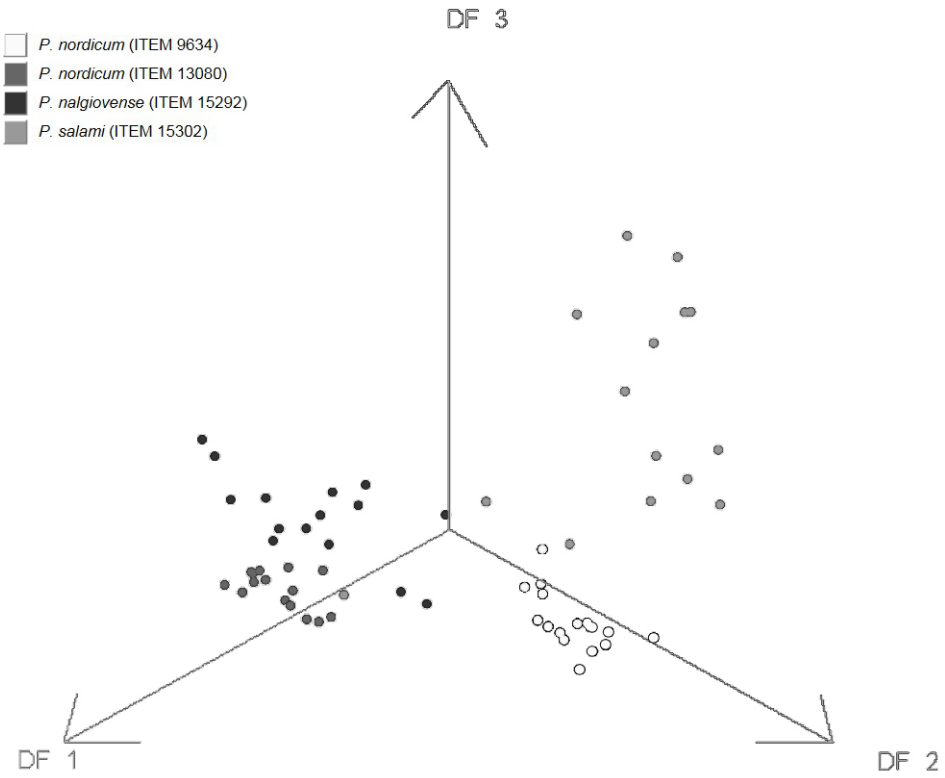

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Figure 2. Pattern of the volatile compounds showing different contents at 5 days of seasoning for dry-cured meat products inoculated with OTA-producer and non-producer strains of *Penicillium*.