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Ecology of moulds during the pre-ripening and ripening of San Daniele dry cured ham

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ABSTRACT

The aim of this work was to study the ecology of moulds and their toxic potential during the pre-ripening or ripening of San Daniele dry cured ham. Three different facilities and their products were investigated. These facilities were chosen on the basis of their production capacity: one for industrial production, one for semi-industrial production and one for handicraft production. The mould microflora was predominantly represented by 2 genera, which were found either on the surface of dry cured ham or in the air of the production rooms. The identified species were quite similar among the three facilities. *Penicillium* spp. and *Aspergillus* spp. were the main genera isolated during the duration of pre-ripening and ripening. The toxic potential of the isolated strains and the presence of Ochratoxin A (OTA) on the surface of or in San Daniele dry cured ham were also investigated. No OTA was found either on the surface (mould slime) or in the meat of the investigated hams. Moreover, no OTA-producing moulds, with the exception of *Aspergillus niger*, were isolated. However, different yeast and mould strains were tested for the ability to inhibit the growth of OTA producing moulds. *Penicillium nalgiovense, Candida guilliermondii* and *Endomycopsis fibuliger* prevented the growth of co-inoculated OTA producing moulds, and no OTA was detected on the surface of the dry cured ham. Thus, it appears that San Daniele dry cured ham does not represent a health hazard.

Keywords: San Daniele ham, mould, Ochratoxin A, *Aspergillus niger*, bioprotective strains

1. Introduction

San Daniele ham is a cured meat product derived from pork thigh that is salted, left to mature, and, over time, acquires aroma and flavour. Historically, the most suitable time period for starting San Daniele dried ham production is from November to February due to good climatic conditions, but modern facilities maintain production all year long.

San Daniele ham is made from heavy pigs (150–220 kg), and the production process includes salting, pressing, drying, pre-ripening and ripening. The ham is completely free of preservatives; stability is linked to salt-induced dehydration and lower water content, which sufficiently limits microbial activity. The entire production process is very long, ranging from 13 to 18 months. During the pre-ripening and ripening stages, the development of mould and yeast growth on the exposed muscle can be very noticeable, in particular when parameters such as temperature and environmental humidity are not maintained. Several biochemical changes are observed, including degradation of fat and proteins by hydrolysis and the development of the characteristic flavour of the meat product. The moulds in particular develop in an uncontrolled manner when the cell produces excessive moisture and condensation accumulates on the surfaces of the product. These moulds derive directly from the product as well as the air environment, and their spread is rapid because they produce mycelial fragments and spores, which are easily carried by air currents and eddies throughout all areas of production, particularly in pre-maturation and ripening areas. The development of moulds during these stages is considered, within certain limits, natural, and they are believed to contribute positively in determining the characteristics typical of the final product (Rojas et al., 1991, Nunez et al., 1996). The moulds are sometimes tolerated in seasoned products because they have antioxidant effects, contribute to the colour (Spotti et al., 2008) and sensory characteristics, prevent excessive drying of the surface, improve the texture and, with proteolytic activity, limit excessive hardness of the flesh (Martin et al. 2006; Sunesen & Stahnke, 2003). Surface moulds of dry cured ham have been studied in great detail (Leistner & Ayres, 1968; Hadlock et al., 1976; Dragoni & Cantoni, 1979; Dragoni et al., 1980a,b; Montel et al., 1986; Huerta et al., 1987; Rojas et al., 1991; Spotti et al., 1988, 1989, 2001a,b; Nunez et al., 1996; Wang et al., 2006; Battilani et al., 2007; Iacumin et al., 2001, 2012). *Penicillium* spp., *Aspergillus* spp. and *Eurotium* spp. seem to be present the most during all stages of ham production (Comi et al., 2004). The prevalence of one genus or another on the surface of industrial hams depends on the ripening temperature, the moisture of the meat and the relative humidity in climatised (controlled) ripening chambers. Conversely, the presence of different moulds on home-made hams is subject to the climate conditions in the production chambers. A considerable number of mouldy areas on the ham

is a sign of improper management of aging conditions and can lead to negative consequences. The development of mould can be related to negative effects such as the production of mycotoxins, which are metabolites that could cause acute an cronic toxic phenomena. The problem of mycotoxins is of primary importance, as they may have carcinogenic, immunologic and allergenic effects, which are particularly relevant in the case of food intake by humans. The developmental capacity of the mould and the possible production of mycotoxins are functions of several interrelated factors, including environmental conditions in particular, which is why temperature and relative humidity must be monitored carefully in ripening rooms. Although the Italian dry cured ham manufacturing process includes operations such as washing, grooming and packaging that allow for the removal of mould, it is possible that abnormous mould development can allow mycotoxin production. Recently, some investigations have highlighted the presence of Ochratoxin A (OTA) in dry cured hams of different origins. OTA is a mycotoxin that is the most important secondary metabolite of several fungi belonging to the genera *Penicillium* and *Aspergillus*. *Penicillium verrucosum, P. nordicum, Aspergillus ochraceus* and *A. niger* can produce OTA when grown on the surface of food, sausages, cured hams and other meat products during ripening and storage (Gareis & Scheuer, 2000, Spotti et al., 2001, 2002; Matrella et al. 2006; Pietri et al. 2006; Comi et al., 2004; Iacumin et al., 2011,2012). However, it is known, even if not always sufficiently demonstrated, that other species of *Penicillium* and *Aspergillus* can produce OTA. Recently, the issue of toxic mould has been the subject of much attention from the food industry. OTA in foods is undesirable because it is classified by IARC (International Agency for Research of Cancer) in "Group B" as a molecule with possible carcinogenic activity in humans. Several studies have shown that OTA has teratogenic, neurotoxic, genotoxic, immunotoxic and nephrotoxic properties (IARC, 1993). For this reason, limits in food have been introduced. In Italy, the maximum concentration of OTA allowed in meat and meat products is 1 mg/kg (Ministry of Health Circular no. 10-09/06/1999).

To our knowledge, there has not been a recent and complete study of the mould ecology of San Daniele dried ham. The aim of this study is to define the mould strains present in San Daniele dry cured ham and in each different type of production facilities to determine their toxic potential.

2. Material and Methods

2.1. Samples

Three San Daniele dry cured ham facilities were chosen on the basis of their production capacity--one with industrial production (A), one with semi-industrial production (B) and one with handicraft production (C)--in order to assess how different production systems, size and management can influence the development of mould and, eventually, the presence of OTA both external and internal to the product.
Samplings were performed every two weeks, from the pre-ripening stage of ham drying to the end of ripening, for a total period of 12 month (24 sampling points).

2.2. Moulds isolation and identification

For the detection of the mould in production areas, an air sampling SAS (SAS - Super 100 - PBI International, Italy) and contact plates with Malt Extract Agar (Oxoid, Italy) were used.
For the detection of moulds on the surfaces of the dry cured ham, contact plates with Malt Extract Agar (Oxoid, Italy) were used. The surface of the muscle without pig skin was sampled.
At the end of ripening, before the sale of the products, surface coatings (slime) by moulds were removed and placed in stomacher bags. Moreover, a portion of meat (0.5 cm) below the slime was sampled by an incision with sterile knives. In this way, approximately 150 samples of slime and 150 pieces of flesh were collected. Part of the slime was used for the detection of moulds and part for OTA determination. Mould colonies grown on Malt Agar (Oxoid, Milano, Italy) were transferred to three different agars--Czapek Dox Agar (Oxoid, Italy), Malt Agar (Oxoid, Milano, Italy) and Salt-Malt agar [5 % malt extract (Oxoid, Italy), 5 % NaCl, 2 % agar (Oxoid, Italy, pH 6.2]--and identified according to Samson & Pitt (2000) and Samson et al. (2004). Some mould strains were identified by molecular methods. Briefly, DNA extraction from pure culture, PCR protocol and identification were performed as reported in Iacumin et al. (2009). The primers were constructed according Kurtzmann & Robnet (1977), and the sequences were aligned in GenBank using the Blast program (Altschul et al. 1997) to determine the closest known relatives of the moulds based on the partial 26S rDNA sequence obtained.

2.3. OTA detection in dry cured ham

The presence of OTA was investigated in the portion of meat taken from 0.5 cm below the slime of the dry cured ham according to the method reported in Comi et al. (2004). Briefly, meat was homogenised, and using aliquots of 10 or 20 g of the homogenate for analysis, OTA was extracted and evaluated by the high performance liquid chromatography (HPLC) method according to Matrella et al. (2006).

2.4. OTA production capability "in vitro"

Different species of moulds that potentially produce OTA were isolated from sausages, and the species belonging to the genera *Penicillium* and *Aspergillus* (1-3 randomly selected strains per species) were tested "in vitro" for the production of OTA by the methods described by Lopez-Diaz et al. (2001) and Paterson & Bridge (1994).

2.5. OTA production capability "in vivo"

The production of OTA was evaluated "in vivo" by inoculating OTA producing strains on meat products. Slices of ham approximately 15 cm^2, including part of the semimembranosus, semitendinosus and biceps femoris muscles, at an Aw of 0.94 (the typical Aw of dry cured ham at the start of the pre-ripening phase) were placed in sterile Petri plates. A suspension of 100 spore/cm^2 of OTA producing species was added to these samples. The suspensions were obtained in peptone water with an activity water (Aw) identical to the substrate and included *Penicillium verrucosum, P. nordicum, Aspergillus niger* and *A. ochraceus*. The plates were kept in the pre-ripening room (R.U. 85-95%, 12-19 °C) for 40 days and then in the ripening room (R.U. 70-80%, 15-22 °C) for 240 days until the end of the ripening for traditional dry cured San Daniele ham. The slime (coating) was removed from the surface, and both slime and meat were tested for the presence of OTA according to the methods reported in section 2.3.

2.6. OTA prevention by bioprotective cultures "in vivo"

The same technique mentioned above (section 2.5.) was used to evaluate bioprotective cultures consisting of *Penicillium nalgiovense, Candida famata* (as suggested by Virgili et al., 2011) and *Saccharomycopsis fibuligera.* In this case, OTA-producing fungi were co-inoculated with suspensions of bioprotective cultures at 100 spores or UFC/cm2. The inoculated meat was incubated and sampled in the same manner as described above (c) to assess the presence of OTA.

3. Results

The mould populations both of the air of the pre-ripening and ripening rooms of three San Daniele dry cured ham plants (A, B, C) and on the dry cured ham were monitored. Samplings were performed every fifteen days, and a total of 24 samplings were performed throughout the year. In total, 40 mould species were identified in the air and on the dry cured ham. Twenty-nine out of 40 species were isolated from both the air and the dry cured ham, 10 out of 40 species were isolated only from the air and 1 out of 40 species was isolated only on the dry cured ham (Table 1). The identified strains were quite similar in all the plants and on all the dry cured ham, demonstrating that contamination mainly came from the air and the seasoning chambers, rather than from the raw meat. The mycoflora was mainly represented by two genera: *Penicillium* spp. and *Aspergillus* spp. Together, these genera represented the 67.6 % of isolates. Among the *Penicillium* spp., *P. chrysogenum* (51 isolates) and *P. citreonigrum* (11 isolates) were the most detected species, while among the *Aspergillus* spp., *A. fumigatus* (55 isolates), *A. flavus* (19 isolates) and *A. niger* (18 isolates) and *A. nidulans* (18 isolates) were detected the most.

No OTA was detected both in slime samples and in the meat of dry cured ham (Table 2). It is likely that the slime samples did not contain any OTA producing moulds. The “in vitro” OTA production of the isolated moulds is shown in Table 3. Only *A. niger* was able to produce OTA “in vitro”. The other strains, as we expected, did not produce OTA. This confirms that only strains of black Aspergilli, *A. ochraceus, P. nordicum* and *P.verrucosum* are able to synthesise OTA either “in vivo” or “in vitro”. Moreover, it has been demonstrated the absence of OTA in the tested slime, considering the slime did never contain the OTA producing strains.

As reported in Table 4, the ability of OTA producers strains to grow on the surface of dry cured ham and produce OTA was demonstrated. It was possible to detect OTA only in the slime produced by growing mould, while no OTA was detected beneath the slime in the meat (5 mm depth). Thus, this result demonstrated that when OTA producing mould grows on dry cured ham, it is possible to detect OTA in the slime. However, OTA could not reach the meat and remained external.

Next, the potential use of bioprotective cultures to limit or prevent the growth of OTA producing moulds, and thus the presence of OTA, on dry cured ham was investigated. The utilised starter cultures completely prevented the growth of the OTA producing moulds. *Penicillium nalgiovense*, *Candida guilliermondii* and *Endomycopsis fibuliger* prevented the growth of co-inoculated OTA producing moulds, and consequently, no OTA was detected on the surface of the dry cured ham (OTA < 0.01µg/kg).

4. Discussion

The identified fungal floras are mainly represented by species belonging to the genera *Aspergillus* and *Penicillium*, which were both found in high numbers in similar studies (Leistner & Ayres, 1968; Bullerman et al., 1969; Hadlock et al., 1976; Dragoni & Cantoni, 1979; Dragoni et al., 1980a, b; Rojas et al., 1991; Spotti et al., 1989; Nunez et al., 1996; Comi et al., 2004; Iacumin et al., 2011,2012). During ripening, a decrease in the *Penicillium* spp. population was noticed, while *Aspergillu*s spp. such as *A. fumigatus, A. flavus* and *A. niger* were more frequently isolated, which has also been reported by other authors (Leistner & Ayres, 1968; Spotti et al.,1989; Nunez et al., 1996; Comi et al., 2004).

Fourteen *Penicillium* spp. were identified, of which 10 were found both in the air and on the hams, 3 were found only in the air and one was found only on the surface of ham. The development of *Penicillium* spp. in the air of the pre-ripening and ripening rooms was observed particularly during the first part of the monitored time period, though it remained fairly constant in plant A. The presence of *Penicillium* spp. decreased during ripening in plants B and C, in agreement with what was reported by Spotti et al. (1989) and Comi et al. (2004). Only the presence of *P. chrysogenum* remained consistent maintained for all ripening times.

This evolution can be explained by the relative humidity in the ripening rooms, which, being higher in the first stage of ripening, may have helped the development of *Penicillium* spp. (Comi et al., 2004).

Penicillium chrysogenum is a halotolerant strain and consequently is one of the strains most commonly isolated among the *Penicillium* spp. It is not a mycotoxigenic strain, and in some studies, it has been shown to improve the quality of the ham by promoting proteolysis (Martin et al., 2006) and to contribute to the development of the typical ham flavour during ripening (Martin et al., 2006). Data obtained from studies of *Penicillium chrysogenum* confirmed that this species is not able to produce OTA “in vitro” and “in vivo” (Iacumin et al., 2009). *P. commune, P. herquei, P. citreonigrum* and *P. diversum* are all species that have been detected only in the drying rooms (data not shown), pre-ripening rooms and in the first two months of ripening. In particular, these species were detected on the surface of hams for up to 60 days and in the air up to 105 days. *P. commune*, which is halotolerant, was detected only in plant B, both in the air and on the hams. This strain has been recognised to be responsible for the phenol acid defect (off-odour). Spotti et al. (1988) demonstrated that only dry cured ham on which *P. commune* had grown presented the phenol acid defect. However, according to the data of the tasters in this study, this defect was not observed. The

presence of *P. citrinum*, which is capable of producing the mycotoxin citrinin according to Sweeney et al. (1998), was rare. *P. citrinum* was detected only once in the air of the plant C 45 days after the beginning of the ripening phase. The presence of *P. spinulosum, P. oxalicum* and *P. purpurogenum* was observed only in the air of the pre-ripening and ripening rooms. In particular, *P. oxalicum* was only detected between the pre-ripening and ripening phases. *P. purpurogenum*, which is halotolerant, has been identified during all of the ripening phase. *P. spinulosum* was detected in all three of the dry cured ham plants, and in particular, at all time points in plant C, as well as during the pre-ripening phase in plant B and at the end of the sampling period in plant A. *P. marneffei* was isolated only in plant B during the initial phase of the sampling and was found on the surface of the ham but not in the air. The presence of *P. rotundum, P. expansum, P. radicum* and *P. verruculosum* extended across the entire sampling period, both on the dry cured ham and in the air. *P. expansum* and *P. verruculosum*, which are moulds capable of producing a mycotoxin that can induce heavy tremors and acute toxicity, were rarely isolated. With regard to the genus *Eurotium*, the only species isolated was *E. amstelodami*. *E. repens*, reported by other authors as a dominant species in cured meat products (Spotti et al., 1989, Nunez et al., 1996, Comi et al., 2004), has never been isolated. *E. amstelodami* was detected on the surface of the hams in factories A and C after two months of ripening, and its presence remained constant until the end of ripening. Comi et al. (2004) had found that the development of strains of xerophilous *Eurotium* spp. on Istrian ham increased during the ripening phase, and these strains rapidly became the dominant genus. This was most likely due to the high resistance of spores to the reduced relative humidity of the ripening room and the dehydration of the surface of the ham. According to what was reported by Spotti et al. (1989), the discovery of strains of *E. amstelodami* growing in dry cured meat is of little importance because is a xerophilous species and has morphological characteristics and environmental requirements very similar to those of *E. repens*. *E. amstelodami* is not a OTA producing mould. Iacumin et al. (2009) has confirmed the inability of this strain to produce OTA “in vitro”.

A total of 8 *Aspergillus* species were identified, the most frequent isolates being *A. fumigatus, A. niger, A. flavus, A. nidulans* and *A. sydowii*. *A. niger, A. fumigatus, A. melleus, A. nidulans* and *A. sydowii* were found both in the air of ripening rooms and on the surface of dry cured hams. *A. fumigatus* was isolated from the dehydration phase up to the first two weeks of ripening. The presence of *A. fumigatus* and *A. flavus* has increased with curing stage advances. Additionally, *A. flavus* and *A. nidulans* are able to produce mycotoxins (Sweeney et al., 1998). However, the potential of toxigenic strains of *A. flavus* has been analysed by Rojas et al. (1991), and it was concluded that the presence of *A. flavus* toxigenic strains on the ham of could not be considered a risk to the health of consumers. *A. flavipes* was only isolated from the surface of the hams in facility

A at the beginning of sampling. Conversely, *A. candidus* was only detected from the air in facility A.

It should also be noted that *Alternaria alternata* strains were present in all three facilities, even though they were not detected continuously during sampling.

Cladosporium cladosporioides is a species that was isolated in all three facilities; its presence in the air was consistent in the first half of the sampling period, while it was only detected on the hams in the first two weeks of ripening. *Cl. herbarum* was isolated on the hams in facility A on the first day of sampling and was present in the air in facilities A and B on day 45 and day 60 of sampling, respectively. This is not a mycotoxigenic strain, as demonstrated by Nunez et al. (1996). The presence of *Cladosporium* spp. is not believed to be important with regard to potential development on the hams in the pre-ripening and ripening phases; the survival of *Cladosporium* spores in the air can be explained by development of this strain on the hams in earlier stages of processing, followed by the transfer of spores from ham to the air (Spotti et al., 1989).

Three species of the genus *Fusarium* were detected. In particular, *F. annulatum, F. acutatum* and *F. solani* were isolated in the air of the three facilities during the pre-ripening stage, while only *F. annulatum* was present on the surface of during pre-ripening and up to 4 months of ripening.

In this work, three strains that are part of the phylum Basidiomycota--*Coprinellus domesticus, Trametes versicolor* and *Phlebia radiata*--were also isolated. The first strain, a fungus with any food value, was isolated in facilities B and C in the first 30 days of sampling. The second strain, which is spread throughout Europe and the world and is known for its colour variability, has been consistently identified both in the air and on the surface of the hams for all three manufacturers. *Leptosphaerulina trifolii* and *Nigrospora oryzae*, which are recognised plant pathogens, were also detected, as well as *Beauveria bassiana*, a fungus used in biological control that can cause damage to various types of insects by acting as a parasite. All are of environmental origin and are derived from outside of the facilities investigated. Typical air species have been occasionally isolated and include *Spaerothryium filicinum, Sporidesmiella fusiformis, Sclerostagonospora opuntiae, Paelomyces variotii, Trichoderma viride* and *Absidia corymbifera* species. The latter is a particular species isolated from plants and soil.

The mycobiota composition detected during this study was not facility-specific. The mould population does not seem to be influenced by plant conditions (temperature, humidity, ventilation, management practices, etc.). Plants A and B have similar technological characteristics and had complete R.U. and temperature control during the pre-ripening and ripening periods. In particular, these plants use R.U.s between 70 and 85 % and temperatures between 12 and 19 °C during the pre-ripening phase and R.U.s between 70 and 80 % and temperatures between 15 and 22 °C during

ripening. Plant C has complete temperature control similar to plants A and B, but it does not have complete R.U. control, and R.U. levels (85 and 90 % during pre-ripening and 75 and 85% during ripening) are higher than in plants A and B. These R.U. differences did not deeply modify the evolution of mould strains. The seasons did not appear to affect species composition. The number and the strains isolated in spring (pre-ripening phase) were quite similar to those isolated in summer-fall (ripening phase). The temperature control in all the investigated facilities most likely did not favour the increased temperature in summer and, consequently, the development of species from the genera *Aspergillus*, *Rhizopus* and *Eurotium*, which are most likely better adapted to a higher temperature. Increased temperature during summer could lead to greater dehydration of the meat products, favouring more xerophilic species of the genus *Eurotium* and in particular *E. amstelodami* (Spotti et al., 1989; Hernández & Huerta, 1993; Castellari et al., 2010; Comi et al., 2004).

All the plants have large processing rooms that are sufficiently ventilated with excellent temperature control. The good ventilation of the pre-ripening and ripening rooms in these plants eliminated condensed water levels on the surface of the dry cured ham (perceptible to the touch), and this environment most likely favoured the establishment of these species and controlled their growth. In fact, independent of the strains, the growth was always light. It was never observed on surfaces of dry cured ham entirely covered by moulds. The slime, which consists of mould mycelium, was observed in spots with 5 mm diameters.

The mould strains isolated in air samples were quite similar to those isolated on dry cured ham, though the number of isolates differed between the air and meat products. The air circulation easily aerosolised spores and permitted their spread in the pre-ripening and ripening rooms, as well as allowing mould strain homogeneity on the dry cured hams. It has been reported that the persistence of high relative humidity and insufficient air circulation in the ripening rooms of some facilities in Croatia was the cause of higher mould prevalence on Istrian dry cured hams (Comi et al., 2004). Mechanical ventilation of the three facilities, which improves air circulation and reduces the relative humidity, could have limited a large amount of mould growth on the dry cured hams. Contamination and growth of toxigenic fungi on dry-cured meat products can result from spore concentration in indoor air, contaminated raw material, unhygienic production rooms and equipment, improper processing and negligence (Asefa et al., 2010; Pitt & Hocking,1999; Kure et al., 2001; Mizakova et al., 2002; FAO, 2003; Battilani et al., 2007; Sorensen et al., 2008). Fortunately, the facilities investigated can prevent and minimise contamination with an integrated approach at all stages of production processes. The GMPs of the producers seem to be sufficient to reduce the level of undesirable mould growth on the product and, in particular, to eliminate the risk

of OTA producing moulds. It was necessary to verify that *Aspergillus* and *Penicillium* lack the ability to produce OTA in vitro because, more and more often, literature demonstrates the presence of OTA in meat products without isolating species which are known OTA producers. In fact, several authors believe that other species can produce OTA (Czerxwiecki et al. 2002; Iacumin, 2009, 2011) and have reported the isolation of low levels of fungi producing OTA associated with high levels of mycotoxin present. In this case, however, only the 19 strains of *A. niger* produced OTA in vitro, and this is consistent with literature data (Samson & Pitt, 2000). All produced OTA "in vitro" but not "in vivo" on dry cured ham; no OTA was found either in the slime produced by the isolated *A. niger* or beneath the slime in the meat (the first 0.5 cm layer). It is possible that the meat is not a good substrate for the production of OTA by the isolated strains of the *A. niger*. In a previous study, Comi et al. (2004) isolated different known mycotoxigenic strains that did not produce the toxic compounds either "in vitro" or "in vivo".

Despite the lack of OTA-producing moulds and OTA on the surface and in the meat of San Daniele dry cured ham, the ability to prevent the growth of OTA producing moulds was investigated. A bioprotective technique was used. The antagonistic potential of yeast and non-OTA producing moulds against toxigenic moulds has been considered for different food matrices (Virgili et al., 2011). They were used as biocontrol agents in cereals (Druvefors & Schnurer, 2005), fruits (Guinebretiere et al., 2000) and in coffee (Masoud &Kaltoft, 2006). The biological control of OTA producing moulds by microbial antagonists to improve food safety without affecting the sensory quality and properties of dry cured ham was investigated (Virgili et al., 2011). In particular, the growth of native yeasts such as *Debaryomyces, Candida* and less frequently *Cryptococcus, Rhodosporidium* and *Rhodotorula*, "in vivo" and "in vitro" seems to inhibit the growth of moulds (Sanchez-Molinero & Arnau, 2008; Wang et al., 2006; Virgili et al., 2011). For this reason, some of these yeast could be used as starter cultures to protect dry cured ham from OTA producing moulds. Moreover, the native yeasts are non-pathogenic, have been shown to be capable of growing to high population levels under conditions, including temperature, pH, moisture, Aw and salt, typical of the dry cured ham surface, and have been used as a starter to improve the volatile compound profile (Martin et al., 2006; Masoud et Kaltoft, 2006; Pinna et al., 2009). In our work, *P. nalgiovense, C. famata* and *E. fibuliger* were utilised to inhibit the growth of ochratoxinogenic moulds (*A. niger, A. ochraceus, P. nordicum, P. verrucosum*) and consequently OTA production on the surface of San Daniele dry cured ham. *C. famata* is a typical native yeast of this meat product (Comi et al., 1980a,b; Simoncini et al., 2007), while *E. fibuliger* was often isolated from the air and from San Daniele dry cured ham (data not shown). *P. nalgiovense* is typically found on dry sausages, and it is used as a starter culture, either to improve quality or as a bioprotective agent for meat products.

Based on this knowledge, it was decided to use these strains as starter cultures to prevent the growth of OTA producing moulds. Our data have demonstrated that these strains completely inhibited the growth of ochratoxinogenic moulds and consequently the presence of OTA. Dry cured ham surfaces were inoculated with both bioprotective strains and OTA producing moulds and were kept at 12-19°C with an R.U. between 75 and 80%. After two weeks, the surfaces were entirely covered by the bioprotective cultures, and the OTA producing moulds were completely inhibited. The inhibition was maintained until the end of ripening (8 months). These data confirm the results obtained in a screen for antagonistic activity against a toxigenic strain of *P. nordicum* and inhibition of OTA biosynthesis conducted by Virgili et al. (2011). According to our data and the data of the above authors, it was suggested that bioprotective cultures might be used to inhibit the growth of OTA producing moulds during pre-ripening and ripening of dry cured hams.

5. Conclusion

San Daniele dry-cured ham is one of Italy's best products. During pre-ripening and ripening, a composite mould population grows on the surface and is present in the air of the production rooms. The main strains isolated are Aspergilli and Penicilli, which predominate either in the air or on the dry-cured ham until the end of the ripening, lasting from twelve to eighteen months. It does not appear that OTA producing moulds can be isolated from the product. In our work, the only potentially toxic strain isolated has been A.niger, which did not produce OTA "in vivo" and only produced OTA "in vitro" on synthetic agar. However, different yeast and mould strains were tested for the ability to inhibit the growth of OTA producing moulds. *Penicillium nalgiovense*, *Candida guilliermondii* and *Endomycopsis fibuliger* prevented the growth of co-inoculated OTA producing moulds, and consequently no OTA was detected on the surface of the dry cured ham.
As of now, it seems that San Daniele dry-cured ham does not represent a health risk for the presence of OTA.

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References

Asefa, D. T., Kure, C. F., Gjerde, R. O., Omer, M. K., Langrud, S., Nesbakken, T., & Skarar, I. (2010). Fungal growth pattern, sources and factors of mould contamination in a dry-cured meat production facility. *International Journal of Food Microbiology, 140*, 131-135.

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 Research, 25*, 3389-3402.

Battilani, P., Pietri, V. 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.

Castellari, C., Quadrelli, A. M., & Laich, F. (2010). Surface mycobiota on Argentinean dry fermented sausages. *International Journal of Food Microbiology, 142*, 149-155.

Comi, G., & Cantoni, C. (1980a). Flora blastomicetica superficiale di insaccati crudi stagionati. Industrie Alimentari, 19, Luglio-Agosto, 563-569.

Comi, G. & Cantoni, C. (1980b). I lieviti di insaccati crudi stagionati. *Industrie Alimentari*, 19, Novembre, 857-860.

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.

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 Additives Contaminants, 19*, 470-477.

Dragoni, I., & Cantoni, C. (1979). Le muffe negli insaccati crudi stagionati. *Industrie Alimentari, 18*, 281-285.

Dragoni, I., Marino, C., & Cantoni, C. (1980a). Muffe in prodotti carnei salati e stagionati (bresaole e prosciutti crudi). *Industrie Alimentari, 19*, 405-407.

Dragoni, I., Ravenna, R., & Marino, C. (1980b). Descrizione delle specie di *Aspergillus* isolate dalla superficie di prosciutti stagionati di Parma e San Daniele. *Archivio Veterinario Italiano, 31*, 1–56.

Druvefors, U.A., & Schnurer, J. (2005). Mould inhibitory activity of different yeast species during airtight storage of wheat grain. *FEMS Yeast Research, 5*, 373-378.

FAO (2008) Risk-based food inspection manual. In FAO, Rome.

Gareis, M., & Scheuer, R. (2000). Ochratoxin A in meat and meat products. *Archivie fur Lebensmittelhygiene, 51,* 102-104.

Guinebretiere, M. H., Nguyen-The, C., Morrison, N., Recich, M., & Nicot, P. (2000). Isolation and characterization of antagonists for the biocontrol of postharvest wound pathogen *Bothrytis cinerea* on strawberry fruits. *Journal of Food Protection, 3,* 386-394.

Kure, C. F., Wasteson, Y., Brendehaug, J., & Skaar, I. (2001). Mould contaminants on Jarlsberg and Norvegia cheese blocks from factories. *International Journal of Food Microbiology, 70,* 21-27.

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. *Journal Clinical Microbiology, 35,* 1216-1223.

Hadlock, R., Samson, R., Stolk, A., & Schipper, M. (1976). Mould contamination of meat products. *Fleischwirtschaft, 56,* 322-327.

Hernandez, E., & Huerta, T. (1993). Evolucion de los parametros microbiologicos del jamon curado. *Microbiologia, 9,* 10–19.

Huerta, T., Sanchis, V., Hernandez, J., & Hernandez, E. (1987). Mycoflora of dry-salted Spanish ham. *Microbiologia Alimentare and Nutrition, 5,* 247–252.

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.

Iacumin, L., Milesi, S., Pirani, S., Comi, G., Chiesa, L. M. (2011). Ochratoxigenic moulds and Ochratoxin A in Sausages from Different Areas in Northern Italy: Occurrence, Elimination or Prevention with ozonated air. *Journal of Food Safety, 31,* 538-545.

Iacumin, L., Manzano, M., & Comi, G. (2012). Prevention of *Aspergillus ochraceus* growth on and Ochratoxin A contamination of Sausages using ozonated air. *Food Microbiology, 29,* 229-232.

I.A.R.C. (1993) Ochratoxin A. In Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. IARC Monograph on the evolution of carcinogenic risks to humans (vol. 56, pp. 489-521) Geneva: International Agency for Research on Cancer.

Leistner, L., & Ayres, J. C. (1968). Moulds and meats. *Fleischwirtschaft, 1,* 62–65.

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. *International Journal of Food Microbiology, 68,* 69-74.

Martín M., Córdoba J. J., Aranda E., Córdoba M. G., Asensi, M. A., (2006). Contribution of a selected fungal population to the volatile compounds on dry-cured ham. *International Journal of Food Microbiology, 110*, 8-13.

Masoud, W., & Kaltoft, C. H. (2006). The effects of yeasts involved in the fermentation of Coffea arabica in East Africa on growth and ochratoxin A. production by *Aspergillus ochraceus*. *International Journal of Food Microbiology, 106*, 224-234.

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. *Czechoslovakia Journal of Food Science, 3*, 89-94.

Montel, E., Villanueva, J. R., & Dominguez, A. (1986). Fungal profiles of Spanish country-cured hams. *International Journal of Food Microbiology 3*, 355–359.

Núñez F., Rodríguez M. M., Bermudez M. E, Córdoba J. J., & Asensio M. A. (1996). Composition and toxigenic potential of the mould population on dry-cured Iberian ham. *International Journal of Food Microbiology, 32*, 185-187.

Paterson, R. R. M., & Bridge, P. D. (1994). Biochemical techniques for filamentous fungi, IMI Technical Handbook, No. 1. CaB International, Surrey, UK.

Pietri, A., Bertuzzi, T., Gualla, A., & Piva, G. (2006). Occurrence of Ochratoxin A in raw ham muscles and in pork products from Northern Italy. *International Journal of Food Science, 1*, 1-8.

Pinna, A., Quintavalla, S., Simoncini, N., Toscani, T., & Virgili, R. (2009) Volatile organic compounds of a ham-like model system inoculated with authoctonous yeasts isolated from typical hams. *Industria delle Conserve, 84*, 91-103.

Pitt, J. I., & Hocking, A. D. (1999). "Fungi and Food Spoilage" 2nd ed. Aspen Publisher Inc. Maryland, USA.

Rojas F. I., Jodral M., Gosalvez F., & Pozo R. (1991). Mycoflora and toxigenic *Aspergillus flavus* in Spanish dry-cured ham. *International Journal of Food Microbiology, 13*, 249-256.

Samson, R. A., & Pitt, J. I. (2000). Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* classification. Harwood Academic publisher, Amsterdam.

Samson, R. A., Hoekstra, E. S., Frisvad, J. C., & Filtenborg, O. (2004). Introduction to Food and Airborne Fungi, Seventh edition. CSB, Wageningen.

Sànchez-Molinero, F., & Arnau, J. (2006). Effect of the inoculation of a starter culture and vacuum packaging (during resting stage) on the appearance and some microbiological and physicochemical parameters of dry-cured ham. *Meat Science, 79*, 29-38.

Simoncini, N., Rotelli, D., Virgili, R., & Quintavalla, S., (2007). Dynamics and characterization of yeasts during ripening of typical Italian dry-cured ham. *Food Microbiology, 24*, 577-584.

Sorensen, L. M., Jacobsen, T., Nielsen, P. V., Frisvad, J. C., & Koch, A. G. (2008). Mycobiota in the processing areas of two different meat products. *International Journal of Food Microbiology*, *124*, 58-64.

Spotti, E., Mutti, P., & Campanini, M. (1988). Indagine microbiologica sul difetto dell'acido fenico del prosciutto durante la stagionatura. *Industria Conserve, 63*, 343-346.

Spotti, E., Mutti, P., & Campanini, M. (1989). Presenza di muffe sui prosciutti durante la prestagionatura e la stagionatura: contaminazione degli ambienti e sviluppo sulla porzione muscolare. *Industria Conserve, 64*, 110-113.

Spotti, E., Chiavaro, E., Pari, E., & Busolli, C. (2001a). Sviluppo di *Penicillium verrucosum* in sistemi modello di prodotti carnei stagionati. Parte II. *Industria Conserve, 76*, 167–183.

Spotti, E., Chiavaro, E., Lepiani, A., & Colla, F. (2001b). Contaminazione da muffe e da ocratossina A in prosciutti stagionati e in fase di stagionatura. *Industria delle Conserve, 76*, 341-354.

Spotti, E., Chiavaro, E. Bottazzi, R., & Del Soldato, L. (2002). Monitoraggio di ocratossina A in carne suina fresca. *Industrie delle Conserve*, *77*, 3-13.

Spotti E., Berni E., & Cacchioli C. (2008). Characteristics and application of moulds. Meat Biotechnology, Toldra (ed.) 181-195.

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

Sweeney M. J., & Dobson A. D. W. (1998). Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *International Journal of Food Microbiology 43*, 141-145.

Virgili, R., Simoncini, N., Toscani, T., Leggieri, M. C., Formenti, S., & Battilani, P. (2011). Biocontrol of *Penicillium nordicum* growth and Ochratoxin A production by Native Yeasts of Dry Cured Ham. *Toxins, 4 (2),* 68-82.

Wang, X. Ma. P., Jiang, D., Peng, Q., & Yang, H. (2006). The natural microflora of Xuanwei ham and the no-mouldy ham production. *Journal of Food Engineering*, *77*, 103-111.

Table 1

Number of isolated strain (in air and on dry cured ham surface) and their identification in the three different monitored plants (A, B, and C).

Genus	Species	Acession Number[1] (GenBank)	Number of isolated strains in air			Number of isolated strains on dry cured ham			Total isolated strains
			Plant						
			A	B	C	A	B	C	
Absidia	*corymbifera*	TM[2]	2	2	-	1	-	-	5
Alternaria	*alternata*	AB363761	4	3	5	3	5	-	20
Aspergillus	*fumigatus*	AB354576	10	5	10	6	15	9	55
Aspergillus	*flavus*	AB363745	3	5	6	1	1	3	19
Aspergillus	***niger***	AF454170	**3**	**3**	**3**	**6**	**2**	**1**	**18**
Aspergillus	*sydowii*	AM883159	1	2	2	3	5	-	13
Aspergillus	*nidulans*	EU840227	6	1	5	3	3	-	18
Aspergillus	*candidus*	EF669609	5	-	-	-	-	-	5
Aspergillus	*melleus*	EF661426	-	1	1	2	-	-	4
Aspergillus	*flavipes*	AB002062	-	-	-	1	-	-	1
Beauveria	*bassiana*	EU334679	-	1	-	-	-	-	1
Cladosporium	*cladosporioides*	EU683047	10	6	6	2	1	-	25
Cladosporium	*herbarum*	EU343661	1	1	-	1	-	-	3
Coprinellus	*domesticus*	AY663837	-	1	2	-	1	-	4
Eurotium	*amstelodami*	AY213699	1	-	2	-	10	5	18
Fusarium	*acutatum*	AY213704	1	-	-	-	-	-	1
Fusarium	*annulatum*	FJ577683	-	1	1	1	1	1	5
Fusarium	*solani*	AY097317	1	-	-	-	-	-	1
Leptosphaerulina	*trifolii*	AY849949	-	-	1	1	-	-	2
Nigrospora	*oryzae*	FJ176892	-	1	-	1	-	1	3
Paecilomyces	*variotii*	AF033395	2	1	1	1	-	-	5
Penicillium	*chrysogenum*	EU862182	16	7	10	3	10	5	51
Penicillium	*citreonigrum*	EF198647	2	2	3	3	1	-	11
Penicillium	*radicum*	DQ981400	2	2	1	1	3	-	9
Penicillium	*herquei*	AF033405	2	1	3	2	-	-	8
Penicillium	*spinulosum*	FJ430767	2	1	5	-	-	-	8
Penicillium	*rotundum*	AF285116	-	1	-	3	3	-	7
Penicillium	*verruculosum*	AF510496	1	1	1	2	2	-	7
Penicillium	*expansum*	AB047232	1	1	1	3	-	-	6
Penicillium	*commune*	AY213617	2	-	-	3	-	-	5
Penicillium	*purpurogenum*	EF087978	2	-	-	-	1	-	3
Penicillium	*diversum*	DQ308554	-	1	1	2	-	-	4
Penicillium	*oxalicum*	AY213620	1	-	1	-	-	-	2
Penicillium	*citrinum*	DQ914650	1	-	1	-	-	-	2
Penicillium	*marneffei*	AB363759	-	-	-	-	1	-	1
Phlebia	*radiata*	AB325676	-	-	2	-	-	-	2

Spaerothryium	*filicinum*	EU552164	1	1	5	1	-	-	8
Sporidesmiella	*fusiformis*	DQ408577	-	-	2	-	-	-	2
Trametes	*versicolor*	AY333793	2	4	5	1	1	2	15
Trichoderma	*viride*	TM	2	1	-	-	-	-	3

Legend: [1]Identification by sequencing and allignment in GenBank as reported in section 2.2; [2]- no isolated strains belonging to this species; [2]Identified by traditional methods.

Table 2

Presence of OTA in slime and in the dry-cured ham meat.

Sample	Number of tested sample	OTA (µg/kg)
Superficial slime	150	< 0.01 (LOD)
Ham	150	< 0.01 (LOD)

Table 3

OTA producers isolates as tested "in vitro".

Genus	Species	Acession Number[1] (GenBank)	Number of isolated strains in air	Number of isolated strains on dry-cured ham	Total isolated strains	% of isolates OTA producers[2]
Penicillium	*chrysogenum*	EU862182	40	27	67	0 %
Aspergillus	*fumigatus*	AB354576	31	18	49	0 %
Aspergillus	*flavus*	AB363745	14	5	19	0 %
Aspergillus	***niger***	AF454170	**9**	**9**	**18**	**100 %**
Eurotium	*amstelodami*	AY213699	3	15	18	0 %
Aspergillus	*sydowii*	AM883159	5	6	11	0 %
Penicillium	*citreonigrum*	EF198647	7	4	11	0 %
Aspergillus	*nidulans*	EU840227	6	3	9	0 %
Penicillium	*radicum*	DQ981400	5	4	9	0 %
Penicillium	*herquei*	AF033405	6	2	8	0 %
Penicillium	*spinulosum*	FJ430767	8	0	8	0 %
Penicillium	*rotundum*	AF285116	1	6	7	0 %
Penicillium	*verruculosum*	AF510496	3	4	7	0 %
Penicillium	*expansum*	AB047232	3	3	6	0 %
Aspergillus	*candidus*	EF669609	5	0	5	0 %
Penicillium	*commune*	AY213617	2	3	5	0 %
Penicillium	*purpurogenum*	EF087978	4	1	5	0 %
Aspergillus	*melleus*	EF661426	2	2	4	0 %
Penicillium	*diversum*	DQ308554	2	2	4	0 %
Penicillium	*oxalicum*	AY213620	2	0	2	0 %
Aspergillus	*flavipes*	AB002062	0	1	1	0 %
Penicillium	*citrinum*	DQ914650	1	0	1	0 %
Penicillium	*marneffei*	AB363759	0	1	1	0 %

Legend: [1]Identification by sequencing and allignment in GenBank as reported in section 2.2; [2]OTA producers were considered that strains able to produce OTA concentration > 0.01 µg/kg (limit of detection - LOD - is 0.01 µg/kg).

Table 4

Isolated strains able to growth and their OTA production capability when intentionally inoculated on dry-cured ham.

Strains	OTA (µg/kg) in meat[1]	OTA (µg/kg) in superficial slime
Penicillium nordicum	1.5	32
Penicillium verrucosum	< 0.01	15
Aspergillus niger	< 0.01	18
Aspergillus ochraceus	< 0.01	28

Legend: [1]meat sampled from a depth of 0.5 cm below the surface; [2]Limit of detection (LOD) is 0.01 µg/kg.