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Moulds contaminants on Norwegian dry-cured meat products

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ABSTRACT

Dry-cured meat production has a long tradition in Norway. However, uncontrolled mould growth on the surface of the dry-cured meat products is causing significant quality problems. As some moulds are mycotoxigenic, their growth on the dry-cured meat products could also pose a serious health risk. Such quality problems and potential health risks can be better handled if the types of moulds growing on the products are known. In total, 161 samples were collected from the ripening and packaging stages of production with the aim of identifying moulds contaminating smoked and unsmoked Norwegian dry-cured meat products. Moulds were isolated either by transferring aerial mycelium of each visible mould colonies on the products or by directly plating pieces of meat on suitable agar media. The isolates were identified at a species level by a polyphasic approach. In total, 264 isolates belonging to 20 species and four fungal genera were identified. The genus *Penicillium* covered 88.3% of the total isolates. This genus contributed to the isolates of smoked and unsmoked products by 91% and 84% respectively. *Penicillium nalgiovense* was the dominant species isolated from both smoked and unsmoked products and covered 38% of the total isolates. *Penicillium solitum* and *P. commune* were the next most frequently isolated species with a contribution of 13% and 10% respectively. Species of *Cladosporium* and *Eurotium* contributed to the total isolates by 6% and 4.9% respectively. Smoking seems to affect the growth of these dominating species differently. An increase in the isolation frequency of *P. nalgiovense* accompanied by the reduction in the occurrence of *P. solitum*, *P. commune* and species of *Cladosporium* was observed on smoked products. The survey showed that the species of *Penicillium* are associated with Norwegian dry-cured meat products in general. *Penicillium nalgiovense*, the dominating mould species, is a potential producer of penicillin and its presence could represent a threat to allergic consumers.

1. Introduction

Dry-cured meat production has been practiced in Norway since the Viking age. At present, several types of dry-cured meat products are industrially produced and the demand for safe products of high quality is increasing. Raw meat develops distinctive flavour as it passes through different processing stages for several months under specific controlled environmental conditions (Rodriguez et al., 1998). Variation in environmental factors at each production stage facilitate unwanted and uncontrolled fungal growth on the surface of the products (Rodriguez et al., 1998). Mould growth on the surface of dry-cured meat products is the result of their tolerance to low pH and high salt concentration (Pitt and Hocking, 1999). Many studies showed that xerophilic species of *Aspergillus*, *Eurotium* and *Penicillium* are associated with dry-cured meat products in different parts of the world (Wu et al., 1974; Monte et al., 1986; Rojas et al., 1991; Nunez et al., 1996; Peintner et al., 2000; Lopez-Diaz et al., 2001; Mizakova et al., 2002; Comi et al., 2004; Tabuc et al., 2004; Wang et al., 2006; Battilani et al., 2007; Papagianni et al., 2007; Sorensen et al., 2008).

The growth of some moulds could be beneficial for the development of characteristic flavour and aroma of dry-cured meat products due to their involvement in the degradation of lipids and proteins. Enzymes of *P. chrysogenum* and *P. nalgiovense* were reported to contribute for lipolytic and proteolytic activities that generated flavour precursors and improved the texture (Rodriguez et al., 1998; Benito et al., 2003, 2005; Martin et al., 2003; Martin et al., 2006). Improvement in the sensory properties of dry-fermented sausages was observed by the effect of *P. aurantiogriseum* in accelerating the production of degraded volatile compounds (Bruna et al., 2001). Moulds have been reported to be involved in anti-oxidation processes

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on dry-cured meat products, which can improve taste, odour and storage quality (Benito et al., 2003; Tabuc et al., 2004; Martin et al., 2006). However, such positive contributions remain unclear. Rather, moulds are frequently referred as cause for food spoilage leading to quality reduction and a major economic loss for the producers. (Filtenborg et al., 1996; Pitt and Hocking, 1999; Samson et al., 2004).

Mould contamination is often associated with unpleasant appearance, odour and changes in taste and nutritional value of foods (Filtenborg et al., 1996; Papagianni et al., 2007). Some moulds are able to produce mycotoxins and antibiotics, which represent a potential health hazard to consumers too. Mycotoxins are secondary metabolites that are toxic to vertebrates when introduced via food through natural route (Samson et al., 2004). Ochratoxin A, which can be immunosuppressive, nephrotoxic, teratogenic, and has been classified as a possible carcinogen to human, has been detected on dry-cured meat products (Chiavaro et al., 2002; Monaci et al., 2005; Toscani et al., 2007). Isolates of *P. commune* from Spanish products produced cyclopiazonic acid on carbohydrate-rich and meat substrates (Nunez et al., 2007). Some strains of *P. nalgiovense* isolated from dry-cured meat products were reported to produce penicillin (Andersen and Frisvad, 1994; Laich et al., 1999; Papagianni et al., 2007). Moreover, thousands of spores can be released from moulds growing on the products in to the air of the production facilities. This can lead to allergic disorders or even chronic lung disease to the staffs of food processing plants (Palmas and Meloni, 1997) in addition to increasing the risk of airborne food contamination.

Identification and characterization of moulds associated with different food products is a starting point to understand their importance. Although dry-cured meat products have been a part of Norwegian food history, the significance of the moulds associated have been paid little attention. The aim of this study was, therefore, to identify moulds associated with the Norwegian dry-cured meat products and get an initial overview on their importance.

2. Materials and methods

2.1. Materials

A total number of 161 dry-cured meat samples were collected from six Norwegian producers in 2007, out of which 110 were at the ripening stage and 51 were fully ripened products. The products sampled were lightly smoked dry-cured hams (103 in total), while the rest 58 were unsmoked Norwegian dry-cured meat specialty called “Fenalår” (dry-cured lamb leg). The age of the dry-cured meat products sampled ranged from 4–22 months, with 53 of them aged more than a year and the rest 108 were younger. The temperature at the ripening stage varied from 12–16 °C. The samples collected have water activity (A_w) of 0.84–0.90.

Sampling from the products at the ripening stage was performed by cutting out pieces of dry-cured meats with visible moulds, while clipped leftovers of fully ripened products were taken during packaging. All samples were forwarded to the mycological laboratory at the National Veterinary Institute without temperature control by overnight mail.

2.2. Mycological procedures

2.2.1. Mould isolation

The isolation of moulds was performed using 9-cm Petri dishes containing Dichloran 18% glycerol (DG-18) (Pitt and Hocking, 1999) agar media. Moulds on samples taken at the ripening stage were isolated by transferring the aerial mycelium of each visible mould colonies at three points on the agar media. A direct plating technique was employed to isolate moulds from the clipped leftovers collected at the packaging stage. The inoculated agar media were incubated for 7 days in dark at 25±1 °C and inspected for genus identification using macro and microscopic morphological characters. The identified genera were then, sub-cultured on suitable agar plates for species identification. Isolates of *Penicillium* were plated on the following media as described in Samson et al. (2004): Malt extract agar (MEA), Czapaek yeast extract agar (CYA), YES (Yeast extract sucrose agar), CREA (Creatine sucrose agar), and NO_2, (Nitrite sucrose agar) and others on MEA and PDA (Potato dextrose agar). MEA, CYA, YES and PDA were incubated in dark at 25±1 °C, while CREA and NO_2 at 20± 1 °C for 7 days.

2.2.2. Mould identification

2.2.2.1. Traditional methods. The mould isolates were identified at species level using a polyphasic approach (Frisvad and Samson, 2004). Macroscopic and microscopic morphological characters were used in the identification process. Colony colour, texture and diameter, the production of diffusible pigments and exudates were among macroscopic features, where as conidia and conidiophore arrangements were the microscopic. To differentiate certain species of *Penicillium*, Erlich test was performed (Frisvad and Samson, 2004). An agar plug was taken out from the center of the colony growing on CYA. A filter paper (1 cm^2) wetted with Erlich reagent was placed on the mycelia side of the plug and checked for the appearance of coloured ring. All the isolates were identified according to Pitt, (Pitt, 1979), Frisvad and Samson (2004), Samson et al. (2004) and Pitt and Hocking (1999).

2.2.2.2. Molecular method: DNA extraction, amplification and sequencing. Molecular identification by sequencing the ITS regions of fungal DNA was performed for some moulds when the traditional method of identification judged to be inefficient. Sequencing was also employed for verifying the identification of the dominant mould isolates. DNA was extracted by Cetyl trimethylammonium bromide (CTAB) DNA extraction protocol (Murray and Thompson, 1980). Shortly, one 4-mm loopful of mould cells from a pure culture was transferred to a 1.5 ml Eppendorf-tube containing 600 μL of CTAB buffer. The cell mixture was frozen at −80 °C for about 20 min, homogenised and resuspended in 400 μL phenol-chloroform. After centrifuging the mixture at 14,000 rpm for 10 min, the upper aqueous phase was transferred to a new Eppendorf-tube. The DNA was then precipitated by adding 300 μL iso-propanol by centrifuging at 14,000 rpm for another 10 min. The DNA pellet was washed once in 70% ethanol, resuspended in 100 μL milliQ water before used as a template.

The fungus-specific universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-GCATATCAATAAGCGGAGGA-3′) were used to amplify genes encoding the ITS region (White et al., 1990). Primers encoding the β-tubulin gene (Glass and Donaldson, 1995), Bt2a (5'- GGT AAC CAA ATC GGT GCT GCT TTC-3') and Bt2b (5'- ACC CTC AGT GTA GTG ACC CTT GGC-3') were used in addition. Ready-to-go-PCR beads (Amersham bioscience, 2003) containing reagents PuReTaq polymerase, 200 μM dNTP (dATP, dCTP, dGTP, dTTP), BSA, stabilizers and reaction buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) were used. PCR was performed in a total reaction volume of 25 μL, which consisted of 2 μL of target DNA solution, 3 μL of each of the primers and 17 μL of milliQ water. The mixture was spinned and set in the PCR machine with the following programmes: an initial denaturation at 95 °C for 10 min, 38 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplification success was evaluated by electrophoresis of the PCR products in 1% agarose gels for 45 min at 80 V in Tris-Borate-EDTA (TBE) buffer. The gels were stained with ethidium bromide, visualized and photographed under UV light. The PCR products were purified by ExoSAP-IT PCR Clean-up Kit (GE Healthcare). Sequencing was performed using an AB1 Prism automated DNA sequencer (Applied Biosystems, Foster city, CA). BigDye Terminator cycle sequencing kit (version 3.1; Applied

Biosystems) was used following the manufacturer's manual on both strands by the same primers. Sequence comparisons were performed using the basic local alignment search tool (BLAST) in GenBank (www.ncbi.nlm.nih.gov/blast) after editing and trimming the sequences by BioEdit sequence alignment editor, version 7.0.0.

2.3. Data analysis

The data collected was analyzed using Microsoft Excel 2002 for Window XP and JMP-6 (version 6.0.0). Descriptive statistics was employed to present the data collected. Each species isolated from each dry-cured meat product sampled was considered as one isolate.

3. Result

In total 264 moulds were isolated from the dry-cured meat samples investigated. The isolates belonged to 20 species of four mould genera. The genera were *Penicillium*, *Cladosporium*, *Eurotium* and *Aspergillus*. The genus *Penicillium* generally dominated the mycobiota of Norwegian dry-cured meat products by covering 88.3% of the total isolates. The genus *Cladosporium* contributed to the total isolates by 6%. The remaining isolates belonged to species of *Eurotium* and *Aspergillus*, which contributed to the total isolates by 4.9% and 0.8% respectively.

Fourteen different species of *Penicillium* were identified out of which 13 of them were from subgenus *Penicillium* (Table 1). *Penicillium nalgiovense* was the most frequently isolated species by covering 38% of the total isolates followed by *P. solitum* and *P. commune* with a contribution of 13% and 10% respectively. Relatively similar isolation frequencies were obtained for *P. chrysogenum*, *P. atramentosum*, *P. crustosum* and *P. brevicompactum*. The four species together contributed to the total mould isolates by almost 21%.

All the four genera were recovered from dry-cured hams (smoked), while three with the exception of *Aspergillus* did from Fenalår (unsmoked). In total, 152 isolates were obtained from smoked products, while 112 were recovered from the unsmoked. The genus *Penicillium* contributed to the isolates of smoked and unsmoked products by 91% and 84% respectively. However, some differences were observed as to the occurrences of the dominant species on smoked and unsmoked products. *Penicillium nalgiovense* dominated the mycobiota of both product types, but its contribution to the total isolates of smoked products (47%) was twice of its contribution to the isolates of unsmoked products (23%). The contribution of *P. solitum* to the isolates of unsmoked products was twice of its contribution to the isolates of smoked products. Similarly, *P. commune* contributed by four folds for the isolates of unsmoked products compared to its contribution to the isolated smocked products (Table 1).

Table 1
Moulds isolated from Norwegian dry-cured meat products

Species	Smoked		Unsmoked		Total isolation frequency
	Ripening	Ripened	Ripening	Ripened	
Aspergillus fumigatus	1	–	–	–	1
A. penicilloides	1	–	–	–	1
Cladosporium cladosporiodes	–	–	5	–	5
C. herbarium	1	–	2	–	3
C. sphaerospermum	–	1	7	–	8
Eurotium amstelodami	4	–	3	–	7
E. herbariorum	5	–	–	1	6
Penicillium atramentosum[a]	12	4	–	–	16
P. brevicompactum	7	3	1	–	11
P. cavernicola	2	–	–	–	2
P. chrysogenum	4	1	11	1	17
P. citrinum	–	–	2	–	2
P. commune[a]	4	2	20	–	26
P. crustosum[a]	4	1	3	6	14
P. discolor	–	–	1	–	1
P. echinulatum	4	–	–	–	4
P. expansum	1	–	–	–	1
P. nalgiovense[a]	46	27	12	14	99
P. palitans	3	–	2	–	5
P. roquefortii	–	–	–	1	1
P. solitum[a]	9	5	12	8	34
Total isolates	108	44	81	31	264

[a] Sequenced.

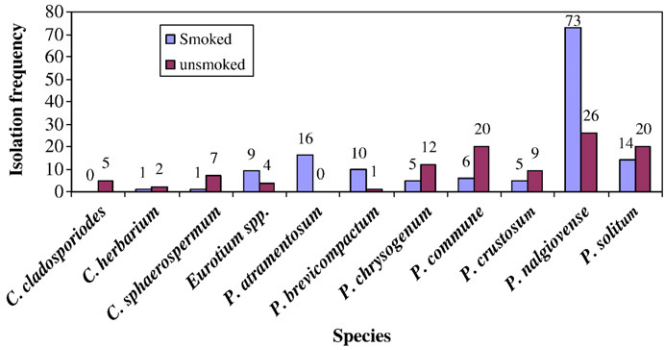

Fig. 1. The occurrence of dominant mould species from smoked and unsmoked dry-cured meat products.

Although *P. nalgiovense* and *P. solitum* were commonly isolated from smoked and unsmoked products, the compositions of dominant species were slightly different. *Penicillium atramentosum* and *P. brevicompactum* were frequently isolated from smoked dry-cured hams, while *P. commune*, *P. chrysogenum* with almost all the species of *Cladosporium* did from unsmoked ones (Fig. 1). The isolation frequencies of *Eurotium* spp. from smoked and unsmoked products were relatively similar. Eight of the isolates of *Eurotium* were recovered from products processed for more than a year.

Lower numbers of species were isolated from fully ripened smocked and unsmoked products compared to the number of species isolated from samples at the ripening stage. Six of the species isolated at the ripening stage were not isolated from fully ripened smoked products, while seven were missing for the unsmoked products. However, this reduction in the total number of species is accompanied by an increase in the occurrence of the dominating species. For example, the contribution of *P. nalgiovense* to the total isolates of smoked products has increased from 44% at the ripening stage to 57% on fully ripened products. The increase for unsmoked products was from 15% to 42%.

In general, moulds were isolated from all the dry-cured meat samples collected. The most frequently isolated species, *P. nalgiovens*, was isolated from 63% of dry-cured hams at the ripening stage and 90% of fully ripened products. The same species was recovered from 32% Fenalår at the ripening stage, while it was isolated from 67% of fully ripened Fenalår.

4. Discussion

Species of *Penicillium* generally dominated the mycobiota of Norwegian dry-cured meat products. *Penicillium nalgiovense*, in particular, covered almost half of the isolated species and seems to be associated with the dry-cured meat products of Norway. Together with *P. solitum* and *P. commune*, the next most frequently isolated species, *P. nalgiovense* have been reported as associated with dry-cured meat products (Filtenborg et al., 1996; Nunez et al., 1996; Peintner et al., 2000; Lopez-Diaz et al., 2001; Sunesen and Stahnke, 2003; Tabuc et al., 2004; Battilani et al., 2007; Papagianni et al., 2007; Sorensen et al., 2008). Frequent isolation of these species have been reported from cheeses too (Lund et al., 1995; Filtenborg et al., 1996; Kure and Skaar, 2000). The tolerance of *P. nalgiovens P. solitum* and *P. commune* to a high level of salt concentration was proved by their

Table 2
Important toxic extrolites that can be produced by the dominating *Penicillium* spp isolated from Norwegian dry-cured meat products

Species	Toxic extrolites	Potential effect
P. atramentosum	– Meleagrin	– Mutagenic
	– Roquefortin C	– Neurotoxic
	– Rugulovasine A	– Anti-hypotensive
P. brevicompactum	– Mycophenolic acid	– Immunosupressive
P. chrysogenum	– Penicillins	– Antibiotic
	– Roquefortine C	– Neurotoxic
	– Meleagrin	– Mutagenic
P. commune	– Cyclopiazonic acid	– Organ damage in mammals
	– Rugulovasine A	– Anti-hypotensive
	– Cyclopaldic acid	– Antibiotic
P. crustosum	– Penitrem A	– Tremorgenic
	– Roquefortin C	– Neurotoxic
	– Terrestric acid	– Cardiotoxic
P. nalgiovense	– Penicillins	– Antibiotic

Source: (Pitt and Hocking, 1999; Samson et al., 2004; Frisvad and Samson, 2004).

growth on dry-cured meat products and cheeses. However, smoking seems to affect the growth of these three species differently. An increase in the isolation frequency of *P. nalgiovense* accompanied by the reduction in the occurrence of *P. solitum* and *P. commune* was observed from smoked product. Smoking is believed to be effective to prevent mould growth on the surface of dry-cured meat products (Food and Agriculture Organization of the United Nations (FAO), 1990), but it looks like that light smoking may not have a preventive effect on the growth of some species like *P. nalgiovense*.

The increasing isolation of *P. nalgiovense* from fully ripened dry-cured meat products, which are ready to be sent to consumers, should be taken seriously as some strains can produce penicillin (Andersen and Frisvad, 1994; Laich et al., 1999; Papagianni et al., 2007). On the other hand, many reported that *P. nalgiovense* is becoming an important starter culture in the production of dry-cured and dry-fermented meat products (Dupont et al., 1999; Lopez-Diaz et al., 2001; Sunesen and Stahnke, 2003; Fierro et al., 2004). Screening strains which are not toxigenic is very important if one considers the use of this species as a starter culture (Rodriguez et al., 1998; Laich et al., 1999; Sunesen and Stahnke, 2003).

A relatively similar isolation frequency of *P. chrysogenum, P. atramentosum*, *P. crustosum* and *P. brevicompactum* in general demonstrated that the associated mycobiota of Norwegian dry-cured meat products is broad. Frequent isolation of *P. chrysogenum* and *P. brevicompactum* were reported from dry-cured meat products of different countries (Wu et al., 1974; Monte et al., 1986; Nunez et al., 1996; Rodriguez et al., 1998; Sorensen et al., 2008). Frisvad and Samson (2004) and Samson et al., (2004), have reported that *P. atramentosum* and *P. crustosum* can contaminate dry-cured meat products and fermented sausages, which was also seen in this study.

All the dominant isolated species of *Penicillium*, with the exception of *P. solitum*, are known producers of toxic secondary metabolites (Table 2) (Samson et al., 2004; Frisvad and Samson, 2004; Rundberget et al., 2004). Their growth on dry-cured meats can possibly lead to the contamination of the products with mycotoxins, which can pose potential health risks. As some people are allergic to antibiotics, like penicillin, the growth of moulds that can release such allergic compounds on the dry-cured meat products can be dangerous for consumers (Laich et al., 1999). The ability to produce toxins and penicillin on products should, therefore, be investigated.

The isolation frequencies of species of *Cladosporium* and *Eurotium* were relatively high. *Cladosporium* spp. seem to be sensitive to smoking as almost all of the isolates were mainly recovered from unsmoked products. *Eurotium* spp have been reported to be among the dominant species of the mycobiota of dry-cured meat products in previous studies (Monte et al., 1986; Comi et al., 2004). Nunez et al., 1996, reported a gradual replacement of *Penicillium* by *Aspergillus* and *Eurotium* in cases of prolonged ripening time. The majority of the *Eurotium* spp were isolated from relatively older dry-cured hams in this study, but reduction in the isolation frequency of *Penicillium* spp. was not observed. The occurrence of species of *Cladosporium* on Norwegian dry-cured meat products agree with studies from Greek and Slovakia (Mizakova et al., 2002; Papagianni et al., 2007). However, in contrast to *Penicillium* and *Eurotium, Cladosporium* is generally not recognized as a genus associated with dry-cured meat products. The species of *Aspergillus* were not common on the Norwegian dry-cured meat products. This may be the result of the climate condition of Norway, which can be too cold for moulds like *Aspergillus* to grow. Species of *Aspergillus* are commonly reported as a part of the mycobiota of dry-cured meat products of countries with warmer climatic conditions (Wu et al., 1974; Rojas et al., 1991; Nunez et al., 1996; Comi et al., 2004).

Generally, species of *Penicillium* were the most important fungi associated with Norwegian dry-cured meat products. *Penicillium nalgiovense, P. solitum, P. chrysogenum, P. crustosum, P. atramentosum, P. commune* and *P. brevicompactum* mainly constitute the associated mycobiota of Norwegian dry-cured meat products. The dominance of the species of *Penicillium* on Norwegian dry-cured meat products could be explained by their ability to grow on products with low water activity and the cold climatic condition of the country.

The present study is the first survey on moulds associated with Norwegian dry cured meat products. Further work should focus on identifying the most important contamination sources and possible preventive measures in the production process. This work can be useful to define the types of toxic metabolites that can possibly be released on the Norwegian dry-cured meat products and their significance to the public health. It can serve as a starting point for the development of non toxic starter cultures, which can be important both for quality improvement and reduction of unwanted fungal growth on dry-cured meat products.

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