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Fungal growth pattern, sources and factors of mould contamination in a dry-cured meat production facility

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ABSTRACT

The aims of this study were to investigate the patterns of fungal growth on dry-cured meat products, identify the important sources and factors of contamination and recommend intervention measures. The production processes of two smoked dry-cured hams and one unsmoked dry-cured leg of lamb were studied. A longitudinal observational study was performed to collect 642 samples from the meat, production materials, room installations and indoor and outdoor air of the production facility. Standard mycological isolation and identification procedures were followed. Totally, 901 fungal isolates were obtained; of which 57% were moulds while 43% were yeast. Yeasts were dominant on meat surfaces by covering 64% of the isolates. Mould growth was not observed until late in the dry-ripening stage. Yeasts and moulds were isolated from half of the environmental samples, of which moulds contributed by 80%. More than 39 mould species were isolated from the entire production process with a 77% contribution by the species of *Penicillium*. *Penicillium nalgiovense* dominated the species composition of moulds isolated from the products and the production environment. A preliminary bioassay analysis on bacterial colonies indicated that most of the *P. nalgiovense* isolates have the ability to produce penicillin. Such isolates might produce penicillin on the products and can become potential food safety hazards. Improper pressing at the salting process, the air quality in salting, brining and smoking rooms and activities in the sorting room were identified as important factors and sources of fungal contamination. Technical solutions and organized production activities that reduce crack formation, airborne spore concentration and improve air circulation in the facility are recommended as intervention measures.

1. Introduction

Dry-cured meat products have been produced and consumed in different parts of the world and significantly contribute to the economies of several countries. The market is increasing and consumers are demanding high quality and safe products free from biological, chemical and physical contaminants. Even if food industries are trying to avoid the contamination of their products via good manufacturing practices (GMP) and standard procedures, a total prevention of contamination is unattainable (FAO, 2008). However, the levels of contaminations can be reduced to an acceptable level.

The growth of microbes, such as bacteria, yeasts and moulds deteriorate the safety and quality of food products and cause significant economic loss (Filtenborg et al., 1996; Pitt and Hocking, 1999; Samson et al., 2004). Fungal particles are the primary causes of spoilage on dry-cured meat products (Rojas et al., 1991; Nunez et al., 1996; Peintner et al., 2000; Comi et al., 2004; Tabuc et al., 2004; Cocolin et al., 2006; Wang et al., 2006; Battilani et al., 2007; Papagianni et al., 2007; Sorensen et al., 2008; Asefa et al., 2009a). Their growth can be facilitated by chains of production processes that each and every process in the chain should be considered to determine where to act (FAO, 2008). Some attempts have been made to identify production factors that facilitate fungal growth on industrial food products. Air has been described as the main source of fungal spores contaminating cheeses (Kure et al., 2001) and dry-cured meat products (Battilani et al., 2007; Sorensen et al., 2008). The quality of raw materials and physical factors like temperature and water activity (a_w) were reported as important factors for fungal growth on the surface of dry-cured meat products (FAO, 1990; Pitt and Hocking, 1999; Mizakova et al., 2002). Manufacturing practices such as salting and smoking are believed to influence the types of fungi growing on dry-cured meat products at the pre-ripening and

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ripening stages (Monte et al., 1986; FAO, 1990; Nunez et al., 1996; Mizakova et al., 2002; Cocolin et al., 2006). The quality of raw materials, the 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).

Undesirable fungal growth may lead to undesirable economic impact to the producers by increasing production cost and product losses. It might represent a potential health hazard to consumers if the fungal contaminants are pathogenic or toxigenic (Mintzlaff et al., 1972; Monaci et al., 2005; Toscani et al., 2007; Iacumin et al., 2009). Such undesirable economic impacts and potential health hazard can be minimized by reducing their growth on the products. This requires the identification of fungi associated with the products, their important sources and the factors facilitating their growth. Fungi associated with Norwegian dry-cured meat products have been reported earlier (Asefa et al., 2009a,c). However, the sources of contaminations and factors facilitating their growth in the overall production processes are missing. This study investigated the patterns of fungal growth on dry-cured meat products, identified the important sources and factors of contamination and recommended intervention measures.

2. Materials and methods

2.1. Sampling process

The production processes of two smoked dry-cured hams and one unsmoked Norwegian speciality "Fenalår" (dry-cured leg of lamb) in a Norwegian dry-cured meat production facility were studied. The experimental design, sampling methodologies, sampling materials and the production processes investigated were similar to the one described in Asefa et al. (2009c). In the production process, fresh and thawed meat pieces were deboned in the trimming room and transferred to the salting room. Before the addition of salt, the meat pieces were pressed and set in elastic net, which kept the pressed meat tight. The pressed meat were dipped in a brine solution before passing through smoking, drying, washing, dry-ripening, sorting and packing processes respectively. Samples were taken consistently from all selected sources at each stage of production. In total, 642 samples were collected in three rounds in 2007, of which 248 were from dry-cured hams, 107 from Fenalår and 287 were environment samples. Air samples constituted 51 of the total environmental samples.

Samples from the surfaces of meat, solid production materials and room installations were taken by wiping sterile wet cotton swabs (Pitt and Hocking, 1999) over a 100 cm^2 surface area. Samples like water, brine solution and salt were taken using sterile 50 ml plastic centrifuging tubes. Small pieces of clipped leftovers of fully ripened products were taken during the packaging process while packaging materials, like net and polyethylene plastics, were taken as part of the environmental samples. Air samples were taken by SAS-super-180 air sampler as described in Asefa et al. (2009b). All the 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. Isolation of fungi

Fungi were isolated using 9-cm Petri dishes containing Dichloran 18% glycerol (DG 18) (Pitt and Hocking, 1999) agar media. Swabs were streaked while 100 µl of liquid samples were spread on the DG 18 plates. Direct plating technique (Pitt and Hocking, 1999) was employed to isolate fungi from salt samples and clipped leftovers of fully ripened products. Packaging materials were stamped on DG 18 plates. The plates, including those used for air sampling, were incubated in darkness at 25 ± 1 °C for 5–7 days before inspection. Mould colonies were identified at a genus level using macro and microscopic morphological characters while yeast isolates were just reported a group. The identified mould genera were 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. Identification

The mould isolates were identified at species level using a polyphasic approach (Frisvad and Samson, 2004), that included both traditional and modern fungal identification techniques.

2.2.2.1. Traditional methods. Macroscopic and microscopic morphological characters were used in the identification process of the mould isolates. To differentiate certain species of *Penicillium*, Erlich and apple test were performed (Frisvad and Samson, 2004). All the isolates were identified according to 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 and β-tubulin regions of fungal DNA was performed for some moulds when the traditional method of identification was judged to be inefficient. Sequencing was also employed for verifying the dominant mould isolates. DNA was extracted by Cetyl trimethylammonium bromide (CTAB) DNA extraction protocol (Murray and Thompson, 1980).

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 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 (Glass and Donaldson, 1995). Similar sample preparation materials, PCR amplification and sequencing protocols described in Asefa et al. (2009c) were used. 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

Descriptive statistics was employed to analyze the data collected using Microsoft Excel 2002 for Window XP. Each species isolated from each sample was considered as one isolate.

3. Result and discussion

3.1. Pattern of fungal growth and dominant mould species in the production process

In total, 901 fungal isolates were obtained from all the samples collected, of which 500 (57%) were moulds and 401 (43%) were yeasts. The meat isolates covered 51% of the total isolates, while 49% were environmental isolates. Yeasts dominated the mycobiota of the meat surfaces from the start to the drying and ripening stages of production. However, their growth was interrupted at the smoking process. The first sign of mould growth on the meat samples was observed at the beginning of the drying and ripening process and their occurrence increased thereafter (Fig. 1). This was noticed on both smoked and unsmoked dry-cured meat production processes. Different food processing stages could facilitate the growth of some selected microbes while hindering others (Fleet, 1999; Cocolin et al.,

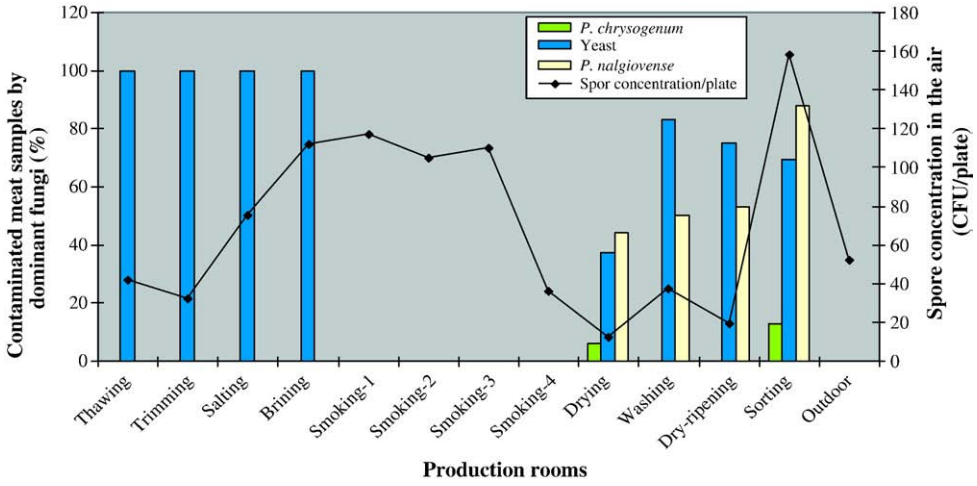

Fig. 1. Dry-cured meat samples contaminated by dominant fungi at each production stage in relation to the mould spore concentration in the air of the production facility.

2006; Hurtado et al., 2008; Asefa et al., 2009c). Yeasts contributed to the total fungal isolates of the meat surface by 64%. The absence of moulds from the start of the production to the start of drying and ripening processes is the main reason for their dominance. This contradicts with previous studies that reported the occurrence of moulds starting from the early stages of production (Nunez et al., 1996; Comi et al., 2004). The occurrence of moulds late in the production process suggests that the sources of contamination are inside the production facility.

Both yeasts and moulds were isolated from the environmental samples at each production stages. However, 47% of the samples taken from room installations and production materials and 2% of the air samples were free from fungal contaminants. Moulds dominated the mycobiota of air samples with a 95% contribution from the start of the production, while yeasts occurred sporadically. The occurrence of moulds on room installations and production materials was limited until the salting process, but increased in a more consistent pattern thereafter. Moulds covered almost 80% of the total environmental isolates (including isolates from the air samples) (Table 1).

The yeast isolates belonged to 10 species of six genera and were described in Asefa et al. (2009c). In comparison, more than 39 mould species were isolated from the entire production facility. Species of *Penicillium* were dominant and contributed by 77% to the total mould isolates followed by *Cladosporium* (6.8%), *Wallemia* (4.6%), *Aspergillus* (2.6%) and *Eurotium* (3.4%). The rest 5.6% was covered by species of *Acremonium*, *Fusarium*, *Mucor*, *Paecilomyces*, *Trichoderma* and sterile fungal colonies (Table 1). The numbers of mould species isolated from the meat surfaces were lower than the number of the species isolated from the production environment. In total, 34 mould species were isolated from the production environment (including air) and the number reduced by more than half on the surface of the meat samples. The surfaces of dry-cured hams harboured 16 mould species, while 11 mould species were isolated from the surfaces of Fenalår. Many of the mould species isolated only once or twice during the survey occurred most likely by chance.

Species of *Penicillium* were isolated frequently from the surface of the meat samples. *Penicillium nalgiovense* dominated followed by *P. solitum*, *P. crustosum* and *P. chrysogenum* on both smoked and unsmoked products of the facility. Some of them are producers of toxic secondary metabolites. *P. nalgiovense* and *P. chrysogenum* are producers of penicillin (Andersen and Frisvad, 1994; Filtenborg et al., 1996) and may cause a potential health problem on people that are allergic to penicillin. A preliminary bioassay analysis as outlined in Andersen and Frisvad (1994) was performed on 58 *P. nalgiovense* isolates to check if they have the ability to produce penicillin. Zones of inhibitions were produced by 54 of the isolates on penicillin sensitive *Staphylococcus aureus* bacterial colonies. These toxigenic isolates may potentially produce penicillin on dry-cured meat products that can represent a food safety hazard. *Penicillium crustosum* produces toxins like penitrems and roquefortin C that can be important food safety concerns (Samson et al., 2004; Frisvad and Samson, 2004; Rundberget

Table 1
Numbers of mould species isolates recovered from the production facility.

Species	Hams	Fenalår	Environment	Air (outdoor)	Total number of isolates
Aspergillus flavus				2	2
A. fumigatus	1		1	1	3
A. niger			1		1
A. penicilloides	1				1
A. sydowii			4		4
A. versicolor			3		3
Acremonium strictum	1	2	3	1	7
Cladosporium cladosporoides[a]			5	5(3)	5
C. herbarum[a]			1	6(3)	1
C. sphaerospermum[a]	1	1	9	6(1)	11
Eurotium amstelodami	2		13	2	17
Mucor circiloniodes		1			1
Penicillium atramentosum	4		1		5
P. brevicompactum[a]	1	1	12	19(3)	14
P. chrysogenum	3	2	9	17	31
P. commune	4		1	1	6
P. cornylophilum			2	6	8
P. crustosum[a]	3	5	10	2(1)	18
P. echinulatum	1		5		6
P. expansum		1		3	4
P. fellutanum				1	1
P. glabrum[a]	2		2	3(1)	4
P. implicatum[a]				2(1)	0
P. italicum				2	2
P. melenii				1	1
P. miczyniskii			1	1	2
P. nalgiovense[a]	52	51	58	43(3)	161
P. olsonii				1	1
P. palitans	1	1	2	1	5
P. raistrickii				1	1
P. roquefortii		1			1
P. solitum[a]	6	8	16	5	35
P. spinulosum			4	2(1)	4
P. tricolour[a]	1		1	2(1)	2
Paecilomyces variotii				1	1
Steril colonies[a]				17(6)	0
Trichoderma sp			1		1
Wallemia sebi			1	22	23
Yeasts	205	107	81	8	401
Total isolates	289	181	247	184	901

[a] Existed in the outdoor air samples too.

et al., 2004). *Penicillium* spp. dominated the mould composition of the environmental samples too. At a species level, *P. nalgiovense* was dominant on the surfaces of room installations, production materials and the air of the production facility. The associated mycobiota of the dry-cured meat facility is generally dominated by species of *Penicillium* specifically *P. nalgiovense*. Associated mycobiota are specific fungi that are capable of growing comfortably and causing spoilage on specific food products (Filtenborg et al., 1996). The association of species of *Penicillium* with dry-cured meat products and production facilities has been described by many previously (Nunez et al., 1996; Palmas and Meloni, 1997; Peintner et al., 2000; Comi et al., 2004; Sorensen et al., 2008; Asefa et al., 2009a).

Even if species of *Penicillium* dominated the composition of environmental isolates, the contribution was not as high as their contribution to the meat isolates. An increase in the occurrence of other mould types at the cost of *Penicillium* spp was observed. For example, *P. nalgiovense* that contributed to the mycobiota of the meat samples by 63% covered only 35% of the isolates of room installations and production materials and 25% of the isolates of the air in the production facility (Fig. 2). This indicates the production environment contains spores of several moulds that were not capable of growing on meat products. However, the production environment was the main source of spores of associated mycobiota. This is in agreement with earlier studies reporting environment as a source of food contaminant moulds (Kure et al., 2001; Mizakova et al., 2002; Battilani et al., 2007; Sorensen et al., 2008).

3.2. Sources and factors facilitating mould contaminants in the production facility

Improper pressing at the salting process, high spore concentration in the air of salting, brining and smoking rooms and unorganized production activities in the sorting room were identified as important factors facilitating the contamination of products by mould.

Visual assessment indicated that most of the dry-cured meat products with visible moulds were those with cracked surfaces resulted from improper pressing. Damaged and undamaged food surfaces were reported to have different microbial ecology which ultimately creates spatial variations in microbial growth (Fleet, 1999). Mechanical damages on the surface of food products were reported to provide suitable microclimate for mould growth (FAO, 2003). Cracks can provide shield to the microbes attached against hygienic activities too (Bower et al., 1996). Hence, the producer must reduce formation of cracks on the product surface either by improving the efficiency of the existing pressing machine or installing new equipment with a better performance. In addition, the operators should have proper training with the technical aspects of the pressing machine and their know-how about the cracked surfaces in relation to mould growth should be enhanced.

The quality of air inside the facility was poor in terms of density and diversity compared to outside the facility. On average 156 colony forming units in a cubic meter of air (CFU/m^3) were obtained from the inside air compared to 118 CFU/m^3 in the outside air. The indoor air samples contained 29 different mould species while only 10 species were isolated from the outdoor. The outdoor air samples were dominated by sterile mycelia and species of *Cladosporium*, where as species of *Penicillium*, especially *P. nalgiovense*, dominated the indoors. Difference in the mycological air quality was also observed among the different production rooms. High spore concentration of the associated mycobiota was observed in the air of salting, brining and smoking room which are located at the center of the production facility (Fig. 1). The rooms were wet all the time and have high level of relative humidity due to insufficient air circulation. High relative humidity and insufficient air circulation have been reported as causes for higher mould prevalence in the central zones of ham producing facilities in Croatia (Comi et al., 2004). Natural or mechanical ventilation that can improve the air circulation and reduce the relative humidity should be in place to bring down the spore concentration in the air of the three rooms (Fig. 1).

Production activities in the sorting room were found to be important in increasing the chance of product contamination by moulds. Right after sorting mouldy and non-mouldy products, moulds were removed either by cutting or scrapping the mouldy product surfaces on open tables. In these processes spores are easily aerosolized as manifested by the highest airborne spore concentration in the sorting room of the production facility (Fig. 1). Thousands of the aerosolized spores can migrate to the neighbouring production rooms as the air pressure gradient in the sorting room was higher. Clear organized activities, physical segregation between clean and unclean areas are of prime importance. It is important to adjust the air pressure gradients, in such a way that the migration of moulds spores from the sorting room to the neighbouring production rooms can be prevented.

In addition, moulds were isolated from the walls and ceilings of salting, brining and washing rooms. Production materials like sticks, net, towel, brushes and trucks contained the spores of the associated mycobiota. They should be subjected the GMP of the producer and attain a satisfactory hygienic qualities.

Contamination and growth of toxigenic fungi on dry-cured meat products can be the results of spore concentration in the indoor air, contaminated raw material, unhygienic production rooms and equipment, improper processing and negligence (Pitt and Hocking,

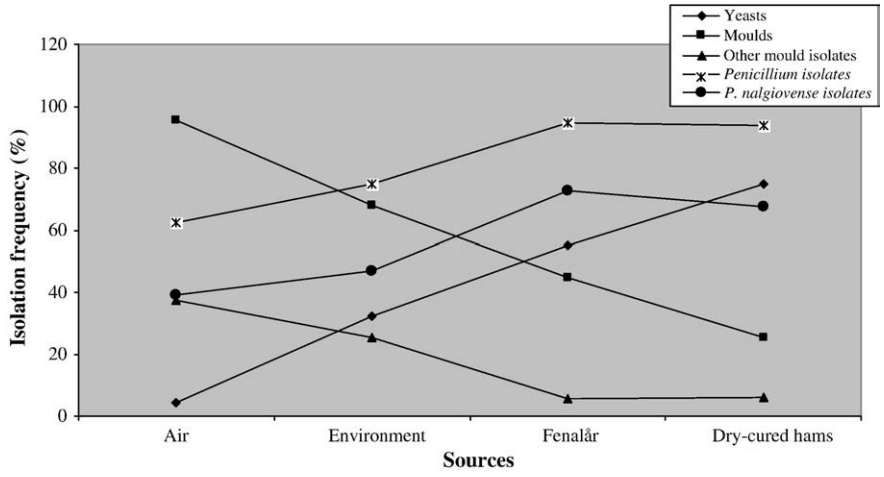

Fig. 2. Occurrences of *Penicillium* spp. in relation to other fungi isolated from air, environmental (room installations and facilities) and meat samples of the production facility.

1999; Kure et al., 2001; Mizakova et al., 2002; FAO, 2003; Battilani et al., 2007; Sorensen et al., 2008). With so many potential sources, contamination cannot be prevented, but only minimized. This needs an integrated approach at all stages of production processes. This study tracked the production processes of dry-cured meat products and identified the sources and factors that facilitate the growth of mould on dry-cured meat products. The GMPs of the producer should address these deviations in order to reduce the level of undesirable mould growth on the product.

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