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The mycobiota of the production environments of traditional Norwegian salted and dried mutton (*pinnekjøtt*)

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ABSTRACT

In 2013, mould growth on Norwegian ready-to-sell *pinnekjøtt* (dried and cured lamb ribs) lead to the withdrawal of 200 tons of the product. The aim of this study was to identify the mycobiota at two Norwegian production sites and determine which species present the highest risk for reduced product quality and safety. A total of 485 samples from 2014, 2015 and 2016 were analysed for *Penicillium* and *Aspergillus* species. Both production sites showed a persistent mycobiota that remained stable over three seasons. Samples from site A were dominated by *P. solitum* while samples from site B were equally dominated by *P. solitum*, *P. brevicompactum/bialowiezense* and *P. nordicum*. The presence of *P. nordicum* was concentrated in one area of the production site where long-time stored hams were produced, but *P. nordicum* was also found sporadically in other parts of the site. Product samples taken from products with visible mould growth were at both sites dominated by *P. solitum*, highlighting its importance for product quality. *P. nordicum* was found frequently in the long-time stored hams, indicating a food safety risk of these products. However, *P. nordicum* was rarely isolated from *pinnekjøtt*. *Aspergillus* spp. were isolated from both sites at all samplings; however, there were no *Aspergillus* isolated from products, and no sites were repeatedly tested positive for identical species, indicating that *Aspergillus* is not a part of the persistent mycobiota, but enters the site sporadically. In conclusion, the study showed that a stable mycobiota consisting of few *Penicillium* species dominated the products and production environments of *pinnekjøtt*.

1. Introduction

Pinnekjøtt is a traditional Norwegian product that is commonly consumed in the fall and winter season and the production season usually spans from August to December. It is produced from lamb ribs and the characteristic flavour and shelf life stability (Leistner, 1992) is developed by salting, storing and drying at ambient temperatures for a prolonged period of time (approximately 14 days at 5–6 °C, 4 days at 18 °C and 14–21 days at 13 °C). Except for salt, no other components are added to the product, and unlike fermented meat products, no starter cultures or other organisms are required to acquire the characteristic flavour. Both smoked and non-smoked variants are produced. As the *pinnekjøtt* production volume varies greatly with seasons, most producers produce *pinnekjøtt* as only one of many products. As a result, production areas are either used for several product types (including other smoked or cured meat products) or in close vicinity to areas where other products are produced. In 2013, the largest Norwegian manufacturer of *pinnekjøtt* encountered major problems with mould growth on their products. Several consumer complaints were registered and as it was initially unknown which moulds were responsible for the contamination of the product, the presence of mycotoxins could not be ruled out, resulting in retraction of 200 tons of *pinnekjøtt* and substantial economic losses. In addition, public awareness was raised by national coverage in Norwegian media (www.nrk.no, www.aftenposten.no, www.tv2.no), resulting in negative publicity for the producer.

Moulds and yeasts are common contaminants on dried and cured meat products as they tolerate both low pH and high salt concentrations (Pitt and Hocking, 1999), and several studies have examined the mycobiota on these types of products (Asefa et al., 2009; Comi et al., 2004; Lopez-Diaz et al., 2001; Sorensen et al., 2008). While some moulds may be beneficial for the flavour and aroma of the products (Martin et al., 2004; Martin et al., 2006; Rodriguez et al., 1998), others may spoil the product. Besides the unappealing appearance of moulded products showing, for example, black spots caused by *Cladosporium* species (Alia et al., 2016; Lozano-Ojalvo et al., 2015) or commonly visible white, green or grey growth by *Penicillium* or *Aspergillus* species (Asefa et al., 2009; Pitt and Hocking, 1999), the production of metabolites may lead to off-odours and –flavours (Pitt and Hocking, 1999; Samson et al., 2004). Also, some moulds can produce mycotoxins or antibiotics which

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may cause health problems in the consumer (Ferrara et al., 2016a; Frisvad et al., 2004). In addition, the extensive production of spores by some fungi may cause allergies or even chronic lung disorders among the production staff (Palmas and Meloni, 1997).

Several Norwegian studies have shown that the mycobiota in dried and cured meats is dominated by various species of *Penicillium* (Asefa et al., 2009; Asefa et al., 2010). These species grow easily on these types of products as they have low requirements for water and grow well at ambient temperatures.

In the aftermath of the 2013 season, this study was conducted to analyse the typical mycobiota in Norwegian production environments for *pinnekjøtt*. The aim was to identify which mould species are present and evaluate their impact on the quality and safety of Norwegian produced *pinnekjøtt*. As species within the genera of *Penicillium* and *Aspergillus* are considered the most important for product safety and quality (Pitt and Hocking, 1999), this study focuses on the identification of *Penicillium* and *Aspergillus* isolates.

2. Materials & methods

2.1. Sampling

Two main producers of *pinnekjøtt* (A and B) were visited during three consecutive production seasons (2014–2016) and a predetermined set of samples was taken at each visit. Both producers produced a range of other products in the same facility, including smoked and non-smoked hams, salami type sausages (both sites, A and B), cured lamb legs and long-time stored hams (site B only), and sampling was carried out in the entire site. Both sites were approximately the same size (60–70 employees), but site A produced a larger amount of *pinnekjøtt* (650 tons per year) compared to site B (250 tons per year). Site A was located close to the coast while site B was located inlands with no proximity to the coastline.

Sampling included air samples by an air sampling device (SAS super-180 air sampler (Bioscience International, Rockville, Maryland)) and standard settle plates, environmental swab samples (VWR Transport Swabs, VWR, Brescia, Italy) as well as raw material, product and water samples.

Where it was possible, air sampler samples and corresponding settle plates were taken in close vicinity to each other. In some cases, no settle plates could be placed due to ongoing activity in the area. The air sampling device was hand held, at least 50 cm away from floors, walls and other obstructions where possible and 500 L were sampled at each site. In in-use smoking and storage rooms, the device was placed as far away from obstructions as possible. Settle plates were left open for 60 min. For all air samples Dichloran 18% glycerol (DG-18) agar (Pitt and Hocking, 1999) plates were used. The plates were incubated in the dark at 25 ± 1 °C for 7 days before inspection. Potential *Penicillium* and *Aspergillus* colonies were harvested with a sterile platinum needle and subcultured on Malt Extract agar (MEA) (Samson et al., 2004) and incubated face up in the dark at 25 ± 1 °C for seven days. If necessary, a second subcultivation was carried out to obtain pure cultures.

Environmental swab samples were taken independently from air samples. Upon arrival at the lab, the swabs were rubbed on DG-18 plates. Material samples, including fresh and used salt, dust and debris, water, slicer waste and meat exudate, were, if possible, distributed directly on DG-18 plates, in addition aqueous solutions of the material samples were prepared and 100 µL were plated on DG-18. All plates were incubated as described for air sampling plates and pure cultures of *Penicillium* and *Aspergillus* obtained as described before.

A total of 485 samples were taken (Table 1). The number of samples varied between visits as production routines changed from season to season. Hence, some rooms that had been sampled previously were no longer in use, while other rooms had been taken in use. In addition, environmental and material samples were chosen at each visit depending among others on the presence of visible moulds on the products and other surfaces.

Table 1
Sampling overview for production sites A and B during production seasons 2014 through 2016.

Sample type	Site A			Site B			Total
	2014	2015	2016	2014	2015	2016	
Air (sampler)	24	25	26	31	31	28	165
Air (settle plates)	20	20	10	28	28	6	112
Swab	18	21	33	17	21	42	152
Material	10	10	17	4	8	7	56
Total	72	76	86	80	88	83	485

No counts were obtained from swab and material samples as these were distributed directly on growth media. For air samples, total mould counts were obtained.

2.2. Mould identification

2.2.1. Morphological identification

Moulds were identified at species level using a polyphasic approach (Frisvad and Samson, 2004). Traditional methods, including macroscopic and microscopic morphological inspection were used. Characteristics like colony size, texture and colour as well as the production of diffusible pigments and exudates on MEA, Czapek yeast extract agar (CYA), Yeast extract sucrose agar (YES), Creatine sucrose agar (CREA) and Nitrite sucrose agar (Samson et al., 2004) were observed and a microscope was used to identify conidia and conidiophore arrangements. Isolates were identified according to accepted keys (Samson et al., 2004; Pitt, 1979).

2.2.2. Molecular identification

Based on their appearance on the selective media, approximately 250 mould isolates were selected for verification of the morphological identification by partial DNA sequencing of the ITS and the β-tubulin region. Strains were cultured on MEA for one week and cell material (one 1 µL loop) was transferred to 600 µL of AL buffer (Qiagen, Hilden, Germany) together with one 3 mm steel bead. The mixture was shaken on a Retsch MM400 shaker (Retsch, Haan, Germany) at 25 Hz for 3 min. Samples were frozen at −80 °C for 10 min and shock thawed at 56 °C. Proteinase K (10 µL, 20 µg/mL) was added and the samples were vortexed and incubated at 56 °C for 30 min. Samples were centrifuged at 12,000*g* for 5 min and DNA was isolated from the supernatant using a QiaCube (Qiagen) and the QIAamp DNA mini QIAcube kit (Qiagen). Sequencing was carried out on the ITS region as well as the β-tubulin region of the genome using a Mastermix consisting of Illustra PureTaq Ready-to-go PCR beads 0,2 mL kit (VWR, Oslo, Norway), miliQ water (17 µL) and primer pairs ITS1/ITS4 (3 mL each) (White et al., 1990) or Bt2a/Bt2b (Glass and Donaldson, 1995), respectively. The total reaction volume was 25 µL. The PCR consisted of an initial denaturation step (10 min at 95 °C), 35 cycles of denaturation (30 s at 95 °C), annealing (20 s at 55 °C for ITS, 58 °C for Bt2) and extension (30 s at 72 °C) and a final elongation step (5 min at 72 °C). PCR products were checked for single products by gel electrophoresis on standard agarose gel, and the PCR products were purified according to the ExoSAP-IT PCR clean-up-protocol (GE Healthcare). Sequencing of the PCR-products of all genes was performed on an Applied Biosystems® 3500xl Genetic Analyzer (Foster City, CA) with BigDye Terminator v3.1 Cycle Sequencing Kit according to standard protocols. Forward and reverse primers used were identical to those previously used in PCR amplification step. All sequencing reactions were purified with BigDye XTerminator Kit before sequenced.

Sequences were edited and trimmed using Geneious R8 (version 8.0.4, Biomatters Ltd.) and compared to known sequences using the local alignment search tools BLAST in GenBank (www.ncbi.nlm.nih.