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Filamentous fungi on meat products, their ability to produce mycotoxins and a proteome approach to study mycotoxin production

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Preface

Mycotoxins are part of our everyday life and can in silence influence our health during long-term exposure. Some mycotoxins can cause or are suspected to promote cancer, a devastating disease for the implicated and their relatives. Although meat products are not a primary food source, they may still contribute to the dietary intake of mycotoxins in certain populations. My intention with the work presented in this thesis was to enlighten the potential health risk of mycotoxin contamination of meat products.

The main part of the study was carried out at Center for Microbial Biotechnology (CMB) at Department of Systems Biology, Technical University of Denmark with supervision by professor Jens Christian Frisvad and associate professor Per Væggemose Nielsen (currently at Institute of Product Development). My sincere gratitude goes to Jens and Per for inspiration, valuable discussions, guidance and support during the study.

I had the opportunity to stay 5 months in the Meat Science research group at Department of Food Science, Faculty of Life Sciences, University of Copenhagen. Here I was introduced to 2D-PAGE based proteome analysis under skilful supervision by associate professor René Lametsch. I am very thankful for the help and support I received by René and the staff in the meat science group.

Certain parts of the study were done in collaboration with the Danish Meat Research Institute, where this Ph.D. study was a part of a project involving several meat processing companies. Programme manager Anette Granly Koch and senior researcher Tomas Jacobsen at the Danish Meat Research Institute are greatly acknowledged for their interest and collaboration, valuable inputs and for supporting with hands-on experiences from the meat industry.

I also wish to thank the technical staff at CMB, Hanne Jakobsen, Jesper Mogensen, Lisette Knoth-Nielsen and Ellen Kirstine Lyhne for great help in the lab, associate professor Kristian Fog Nielsen for invaluable guidance in analytical chemistry and post doc. Mikael Rørdam Andersen for great inputs to *Aspergillus niger* protein annotation. I also appreciate my colleagues and fellow PhD students for making CMB a nice place to stay.

Finally I like to thank my family and friends and especially Dennis for encouragement, support and patience during the past years.

Marie Sørensen

June 2009, Kgs. Lyngby, Denmark.

Abstract

Mould growth can occur on meat products like dry-cured hams, fermented sausages and pâtés. Since many species of filamentous fungi are able to produce mycotoxins, this raises the question if uncontrolled mould growth on meat products is a potential food safety risk. The main objective of this study was to determine if filamentous fungi from meat processing environments can produce toxic secondary metabolites during growth on meat products and to enhance the understanding of conditions that can influence the production.

Initially the mycobiota of four North-European meat processing plants used for production of either fermented sausages or liver pâtés was investigated. The main genera were *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* and *Phoma*. While *Penicillium* was isolated frequently at all four processing plants, *Cladosporium* was isolated frequently in processing plants examined in the autumn and *Eurotium* was isolated frequently at one plant producing fermented sausages. Sixteen *Penicillium* species were identified including a new species named "*P. milanense*" (ined.). At least half of the *Penicillium* species were potentially able to produce toxic secondary metabolites. The most frequent were *P. brevicompactum* and the closely related *P. bialowiezense* both producing the immunosuppressive drug mycophenolic acid and *P. palitans* producing the mycotoxin cyclopiazonic acid.

Next, *P. brevicompactum* was used to inoculate hams, sausages and liver pâtés that were processed in a pilot plant facility. An analytical method for quantitative determination of mycophenolic acid in meat products was developed, which was based on extraction at basic pH followed by mixed mode reversed phase-anion exchange and LC-MS. The limit of detection was 4 µg/kg in sausage and 6 µg/kg in ham and pâté. Mycophenolic acid was detected in dry-cured hams and liver pâtés, but not in fermented sausages. Levels ranged from 190 µg/kg in centre to 11 mg/kg in surface of hams and from 150 µg/kg in bottom to 14 mg/kg in surface of liver pâtés.

From these studies it was concluded that the mycobiota of meat processing environments include toxinogenic species and that one of these, *P. brevicompactum*, was able to produce mycophenolic acid on meat products like dry-cured ham and liver pâté during realistic processing conditions.

Last, proteome analysis was used to reveal cellular mechanisms that influence mycotoxin production. *Aspergillus niger* produces two important mycotoxins, ochratoxin A and

fumonisin B_2, and was used as model organism since its genome sequence is known, which facilitates identification of proteins, as well as it is a species that have been isolated from meat products in several previous studies. Combination of the carbon sources starch and lactate in the substrate, that both are present in certain types of fat-reduced fermented sausages, was found to increase production of fumonisin B_2 synergistically compared to either carbon source alone. A 2D-PAGE based proteome analysis was used to find proteins with levels that correlated with fumonisin B_2 production on media containing 3 % starch, 3 % starch + 3 % lactate and 3 % lactate. These proteins were identified using MALDI TOF/TOF mass spectrometry and were mainly enzymes involved in the pentose phosphate pathway, the tricarboxylic acid cycle, ammonium assimilation and oxidative stress. Many of these enzymes are expected to influence the intracellular levels of acetyl-CoA and NADPH. Thus, the results indicated that high levels of acetyl-CoA and abundance of NADPH correlated with high fumonisin B_2 production. In this case, proteome analysis was used successfully to give an idea of how starch and lactate influence fumonisin B_2 production by *A. niger* and may be a valuable tool in future studies to enhance the understanding of mycotoxin production by filamentous fungi.

Dansk resumé

Skimmelsvampe på kødprodukter, deres evne til at danne mykotoksiner og brug af proteomanalyse i studier af mykotoksin produktion

Skimmelsvampe kan forekomme på kødprodukter som spegeskinker, spegepølser og postejer. Da mange arter af skimmelsvampe kan danne mykotoksiner, rejser dette spørgsmålet om, hvorvidt ukontrolleret skimmelvækst på kødprodukter er en fødevarerisiko. Det overordnede formål med dette projekt var at bestemme, om skimmelsvampe fra kødforarbejdningsmiljøet kan producere toksiske sekundære metabolitter under vækst på kødprodukter og at øge forståelsen af forhold, der kan påvirke produktionen.

Indledende blev mykobiotaen undersøgt på fire nordeuropæiske kødforarbejdsningssteder anvendt til produktion af enten spegepølser eller leverpostej. De primære slægter var *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* og *Phoma*. Mens *Penicillium* blev isoleret hyppigt fra alle fire forarbejdningssteder, blev *Cladosporium* hyppigt isoleret fra forarbejdningssteder undersøgt om efteråret, og *Eurotium* blev hyppigt isoleret fra et forarbejdningssted anvendt til spegepølseproduktion.

Seksten *Penicillium* arter blev identificeret inklusiv en ny art kaldet "*P. milanense*" (ined.). Mindst halvdelen af *Penicillium* arterne var potentielt i stand til at danne toksiske sekundære metabolitter. De hyppigst forekommende var *P. brevicompactum* og den tæt beslægtede *P. bialowiezense*, der begge producerer det immunhæmmende stof, mykophenolsyre, og *P. palitans*, der producerer mykotoksinet cyclopiazonsyre.

Dernæst blev *P. brevicompactum* brugt til at pode skinker, pølser og leverpostejer, som blev fremstillet i et pilotanlæg. En analytisk metode til kvantitativ bestemmelse af mykophenolsyre i kødprodukter blev udviklet, som var baseret på ekstraktion ved basisk pH efterfulgt af mixed mode revers fase - anionbytning og LC-MS. Detektionsgrænsen var 4 µg/kg i spegepølse og 6 µg/kg i skinker og leverpostej. Mykophenolsyre blev detekteret i spegeskinker og leverpostej, men ikke i spegepølser. Niveauerne var mellem 190 µg/kg i centrum til 11 mg/kg i overflade af spegeskinke og mellem 150 µg/kg i bunden til 14 mg/kg i overfladen af leverpostej.

Ud fra disse studier blev det konkluderet, at toksinogene skimmelsvampe er blandt mykobiotaen i kødforarbejdningsmiljøet, og at en af disse, *P. brevicompactum*, kan producere mykophenolsyre på kødprodukter som spegeskinker og leverpostej under realistiske forarbejdningsforhold.

Til sidst blev proteomanalyse benyttet til at belyse cellulære mekanismer, der påvirker mykotoksinproduktion. *Aspergillus niger* producerer to vigtige mykotoksiner, ochratoksin A and fumonisin B_2, og blev brugt som modelorganisme, idet genomsekvensen er kendt, hvilket gør det lettere at identificere proteiner, og fordi det er en art, som er blevet isoleret fra kødprodukter i flere tidligere studier. Kombination af kulstofkilderne stivelse og laktat i mediet, som begge forefindes i visse lavfedtholdige kødprodukter, blev fundet at forøge produktionen af fumonisin B_2 synergistisk i forhold til hver af kulstofkilderne alene.
En 2D-PAGE baseret proteomanalyse blev anvendt til at finde proteiner hvis niveauer korrelerede med fumonisin B_2 produktionen på medier med 3 % stivelse, 3 % stivelse + 3 % laktat and 3 % laktat. Disse proteiner blev identificeret ved brug af MALDI TOF/TOF masse spektrometri og var primært enzymer involveret i pentose phosphat vejen, tricarboxylsyre cyklussen, ammonium assimilering og oxidativt stress. Mange af disse enzymer forventes at påvirke det intracellulære niveau af acetyl-CoA og NADPH.
Resultaterne indikerede derfor, at høje niveauer af acetyl-CoA og rigelige mængder af NADPH korrelerede med høj fumonisin B_2 produktion. I dette tilfælde blev proteomanalyse anvendt succesfuldt til at få en ide om, hvordan stivelse og laktat påvirker fumonisin B_2 produktion hos *A. niger,* og kan være et værdifuldt redskab i fremtidige studier til at forøge forståelsen af skimmelsvampes mykotoksinproduktion.

List of original manuscripts

Manuscript 1

Sørensen LM, Jacobsen T, Nielsen PV, Frisvad JC, Koch AG: Mycobiota in the processing areas of two different meat products. International Journal of Food Microbiology 2008, 124:58-64.

Manuscript 2

Sørensen LM, Nielsen KF, Jacobsen T, Koch AG, Nielsen PV, Frisvad JC: Determination of mycophenolic acid in meat products using mixed mode reversed phase-anion exchange clean-up and liquid chromatography-high-resolution mass spectrometry. Journal of Chromatography A 2008, 1205:103-108.

Manuscript 3

Louise M Sørensen, Rene Lametsch, Mikael R Andersen, Per V Nielsen, Jens C Frisvad: Proteome analysis of *Aspergillus niger*: Lactate added in starch-containing medium can increase production of the mycotoxin fumonisin B_2 by modifying acetyl-CoA metabolism. BMC Microbiology 2009, 9:255.

Contents

Introduction

Some species of filamentous fungi are able to grow at relatively low water activities and are thus able to grow on meat products that have been preserved against bacterial spoilage by salting and drying [1]. Of special importance is species belonging to the genera *Aspergillus* and *Penicillium* as many of these species can produce mycotoxins [2]. For traditional reasons there are different views on presence of mould growth on meat products. Moulds play an important role in the ripening of some meat products in several South-European countries as Italy, Spain, France, Hungary and Southern Germany. These mould-fermented products are characterised by the presence of a white and occasionally green mould cover on the surface and are appreciated due to development of characteristic taste, flavour, texture and appearance [3]. While inoculation has traditionally been done with the indigenous mycobiota of the processing plants, toxicologically safe commercial mould starter cultures are of common use today at industrial-scale plants. In most North-European countries including Denmark mould growth is not tolerated on meat products. Preservation by salting and drying, fermentation and/or smoking is commonly used to control mould growth and after ripening often combined with storage at cooling temperature or with modified atmosphere packaging. Salting and drying typically lower the water activity to between 0.80-0.95 in products like fermented, dry sausages and dry-cured ham [1], while fermentation of sausages lowers the pH to 4.5 – 5.0 [4]. The smoking process includes a mild heat treatment, but most importantly antimicrobial compounds from the smoke are absorbed to the surface of the products [5]. While each of these treatments alone can not prevent fungal growth, the combined use has generally been sufficient to protect meat products. However problems arise when the conditions are not controlled properly. Typical problems are inadequate fermentation leading to a slow pH decline, variations in the smoking process or uncontrolled fluctuations in temperature and humidity during drying and ripening. Also unusual high spore concentrations for example after rebuilding or from the outside air may lead to a higher incidence of mould contamination. Loss of valuable meat products as dry-cured hams due to mould growth can be a significant economic burden for meat manufactures. Thus it is a common procedure to remove mould spots by brushing, washing or cutting it away at some producers [6]. This despite little is known about the ability of filamentous fungi to produce mycotoxins during growth on meat products and about diffusion of mycotoxins into the products. However, there is in general an increasing awareness about these problems in the meat industry. For health

reasons there is also an interest in the meat industry to lower the salt and fat content of products and to replace smoking with the use of smoke aromas [5]. Such changes in the production may lead to enhanced problems with mould growth and may influence the mycotoxin production. The present project serves to enlighten these topics. Thus, the **primary objective** of this study was to determine if filamentous fungi from meat processing environments can produce toxic secondary metabolites during growth on meat products and to enhance the understanding of conditions that can influence the production. The study consists of three parts (figure 1).

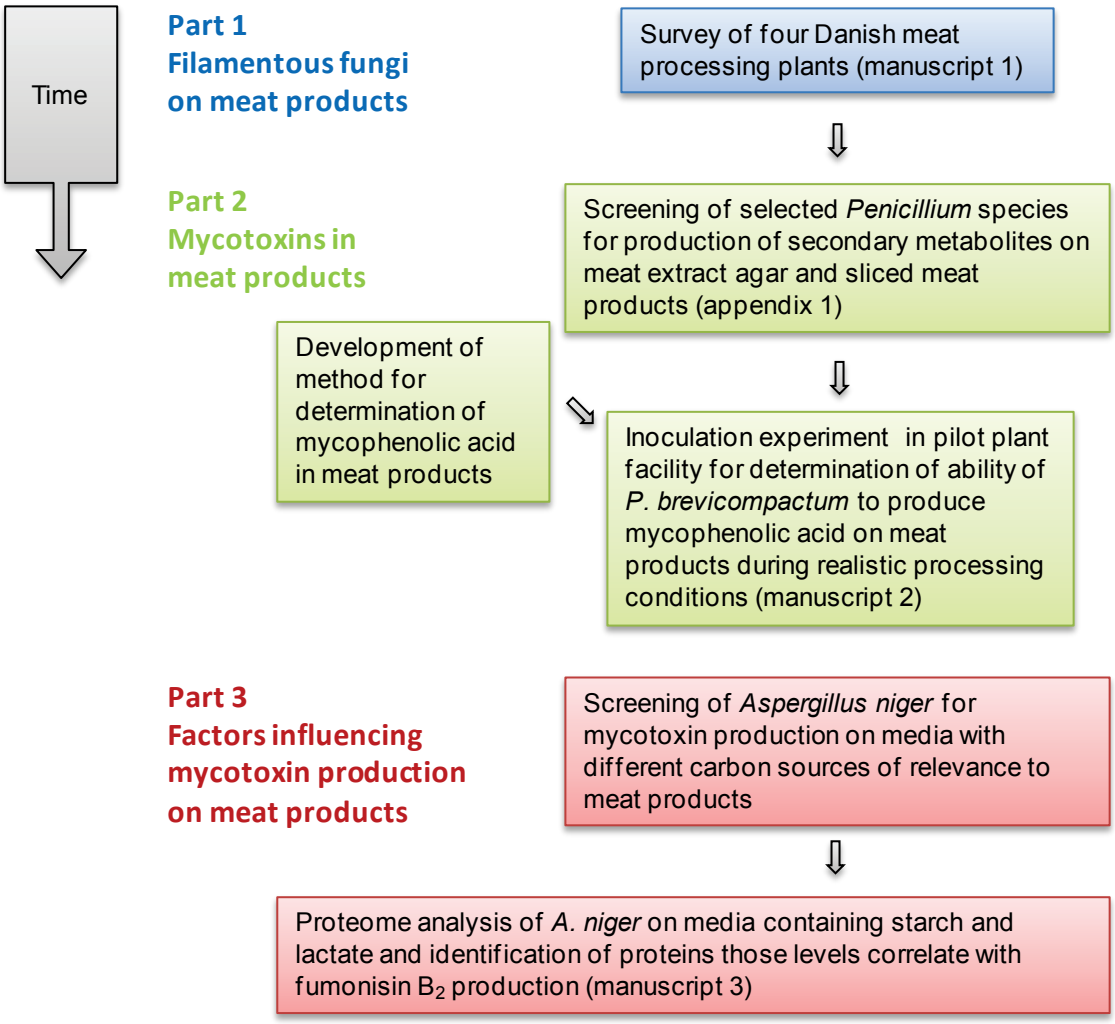

Figure 1: Overview of the study and the experimental work.

In the **first part** it was the aim to establish the mycobiota of Danish meat products and determine the presence of toxinogenic fungi. The results from a survey of four meat processing plants are presented in manuscript 1. In the **second part**, the aim was to determine if species from the mycobiota of Danish meat processing environments can produce secondary metabolites on meat products during realistic processing conditions and if the metabolites penetrate into the meat products. Species producing mycophenolic acid and cyclopiazonic acid were prevalent in the processing plants and thus the ability of these species to produce secondary metabolites on meat extract agar and slices of purchased meat products was tested preliminary (results are shown on a poster in appendix 1). Since *P. brevicompactum* was a consistent producer of mycophenolic acid (MPA) on sliced meat products and was present in all four surveyed meat processing plants, this species was selected for an inoculation study of three different types of meat products in a pilot plant facility. To be able to analyse the meat products for presence of MPA, an analytical method was developed for this. The method and the results from the inoculation experiment are presented in manuscript 2. The aim of the **third part** was to gain knowledge, at a cellular level, of how mycotoxin biosynthesis is affected by conditions that influence production of mycotoxins. The experimental approach was based on the hypothesis that changes in conditions that cause a profound effect on secondary metabolite production also lead to changes in the protein expression. Combination of physiological studies of growth and secondary metabolite production with proteome analysis can then potentially be used to reveal cellular mechanisms affecting the secondary metabolism (approach illustrated in figure 2).

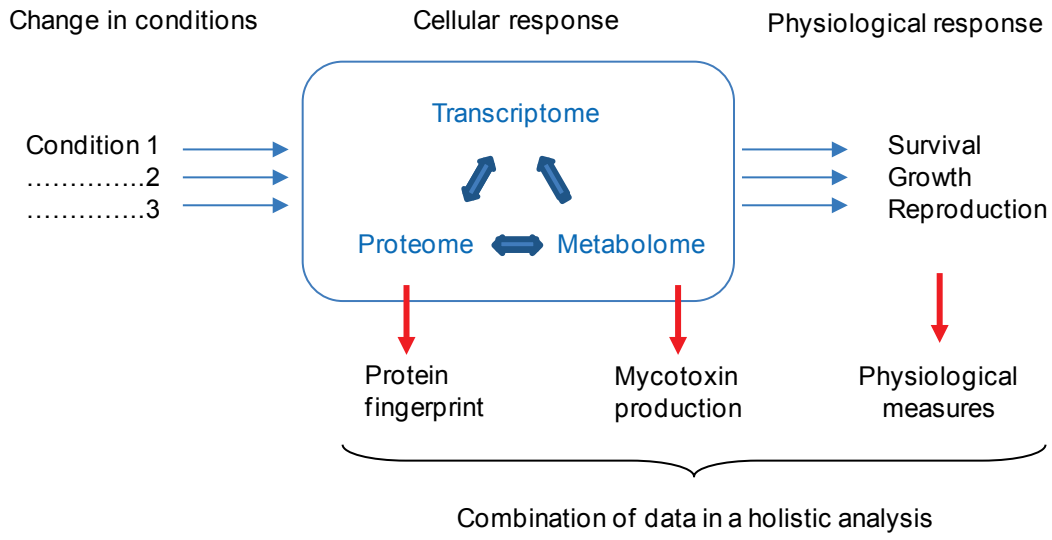

Figure 2: Experimental approach for the work in part 3 (manuscript 3).

The proteome analysis was based on 2D PAGE followed by identification of selected proteins by MALDI TOF/TOF tandem mass spectrometry. Availability of the whole genome sequence for the species studied greatly facilitates identification of proteins [7]. For that reason *A. niger* was used as a model organism since it, among the sequenced species, is frequently isolated from meat products [8-16] as well as it can produce two important mycotoxins; ochratoxin A (OTA) and fumonisin B_2 (FB_2) [17,18]. A huge number of factors may influence mycotoxin production on meat products during processing and storage. Availability of nutrients is known to have an enormous influence on mycotoxin production by some species [19-21]. Glucose is usually added to sausages for support of the bacterial starter cultures applied, but this carbon source is utilised rapidly during fermentation accompanied by formation of lactic acid and other fermentation products [4]. However, potato starch is added to some types of fermented sausage products in order to lower the fat content. Addition of such readily utilisable and energy-rich carbon sources to meat may be expected to have a large effect on the potential mycotoxin production. Thus the effect of carbon sources as glucose, starch, fat and lactate and a few combinations hereof on OTA and FB_2 production by *A. niger* was screened in order to identify conditions with a large influence on mycotoxin production. The results of this and the application of proteome analysis to elaborate on how the mycotoxin production was affected at the cellular level are presented in manuscript 3.

The three manuscripts included in this thesis are compiled in the three following chapters. Each of the manuscripts is preceded by a brief background section describing some of the existing knowledge, important literature and other information relevant for setting the manuscripts in perspective. After this, the most important results are summarised and discussed in terms of implications and perspectives of the obtained results. Finally the thesis is ended with the most important conclusions of industrial and scientific interest.

Part 1 Filamentous fungi on meat products

Background

Presence of filamentous fungi on meat products as fermented sausages and dry-cured hams has been the subject of several studies of various geographical origins and with an increasing attention within the last 50 years (figure 1). The topic has been reviewed by Leistner and Eckardt in 1981 [22] and mentioned briefly in later publications [4,23-25].

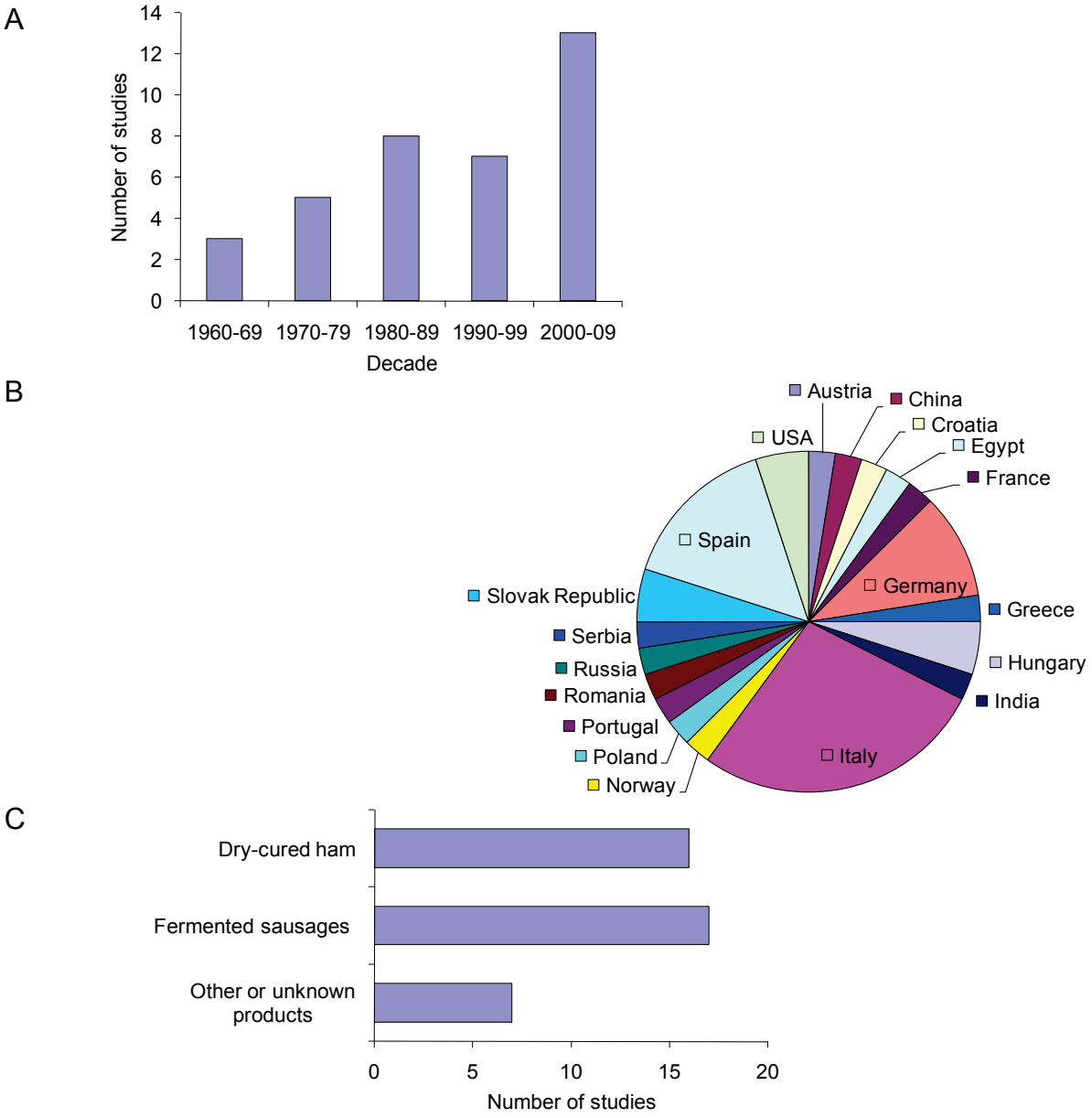

Figure 3: Studies reporting filamentous fungi on meat products.
A) Published year, B) geographical origin of products, C) type of products.

Filamentous fungi reported in studies of fermented sausages [12,15,26-40], dry-cured hams [8,13,14,16,32,40-50] and other, unknown or unspecified products [10,11,41,45,51-53] are listed in table 1. Frequently reported genera were *Alternaria*, *Aspergillus*, *Cladosporium*, *Eurotium*, *Mucor*, *Penicillium*, *Rhizopus* and *Scopulariopsis*. The genus *Penicillium* was reported within all examined studies. Besides being ubiquitously present, *Penicillium* spp. were often dominating on fermented sausages [12,26,34,35,37] or were a significant part of the mycobiota together with *Scopulariopsis* [31], *Aspergillus* [15,29] or *Eurotium* [30], but were only in a few cases dominating on dry-cured hams [41,42]. The mycobiota of dry-cured hams was more often dominated by *Aspergillus* spp. [8,14,50] or *Eurotium* spp. [13,44,46,48,49]. Some *Aspergillus* spp. and especially *Eurotium* spp. are xerophilic and are thus able to grow at the low water activities (< 0.80) [25] that occur on the surface of dry-cured hams at the end of ripening. Nunez et al. [13] observed that while *Penicillium* spp. were dominating initially after salting and during ripening, *Aspergillus* and *Eurotium* spp. became dominant when the surface water activity of the hams dropped below 0.88-0.79.

The genus *Penicillium* was represented by a very large number of species (table 2). Several revisions of the taxonomy during the years complicate the compilation of reported species, but corrections or synonyms to the names of the species listed in the original articles were used if appropriate. In addition, it is most likely that the list in table 2 to some degree is inaccurate due to misidentification of isolates [54]. Many species, especially the food-related terverticillate *Penicillium* spp., are morphologically very similar and identification is not trivial. In some of the recent studies, secondary metabolite profiles and/or molecular techniques were used to supplement the morphological and physiological characteristics and thus gave more confident identifications [30,37,41]. Frequently reported *Penicillium* species were *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. crustosum*, *P. cyclopium*, *P. commune*, *P. expansum*, *P. nalgiovense*, *P. roqueforti*, *P. solitum*, *P. verrucosum* and *P. viridicatum*. In addition *P. olsonii* was frequently reported from fermented sausages, otherwise there was no obvious distinction within *Penicillium* spp. between the product types. The frequent occurrence of these *Penicillium* spp. on meat products is probably due to their common ability to tolerate low water activity (0.78-0.83) and to grow well at low to moderate temperatures and on protein-rich substrates [22,25,55]. Dominating species were typically *P. nalgiovense* [26,30,41,42], *P. solitum* [37] and *P. commune* [31,34].

Table 1: Filamentous fungi isolated within mycological studies of meat products.
Isolated genera, number of studies within it is isolated and references.

Genera	Number of reporting studies					
	Fermented sausages		Dry-cured hams		Other meat products	
	No.	Ref.	No.	Ref.	No.	Ref.
Ascomycota						
Acremonium	1	[35]	-		2	[10,51]
Acrostalagmus	1	[38]	-		-	
Alternaria	5	[15,29,30,37,39]	6	[8,13,14,16,32,50]	3	[10,11,51]
Aspergillus	12	[12,15,26,28-32,35,36,38,39]	11	[8,13,14,16,32,41-44,49,50]	4	[10,11,51,53]
Aureobasidium	1	[37]	2	[13,14]	-	
Botrytis	1	[15]	1	[16]	1	[53]
Chaetomium	-		-		1	[53]
Cladosporium	8	[12,15,26,28,29,37-39]	6	[8,13,14,32,41,50]	5	[10,11,41,51,53]
Clasterosporium	-		1	[16]	-	
Colletotrichum	-		-		1	[10]
Curvularia	-		1	[13]	1	[10]
Doratomyces	-		-		1	[53]
Emericella[1]	1	[38]	2	[14,50]	1	[10]
Epicoccum	1	[29]	1	[32]	1	[10]
Eupenicillium[2]	1	[30]	1	[32]	-	
Eurotium[1]	6	[26,29,30,32,35,38]	11	[8,13,14,32,41,44,46-50]	2	[41,51]
Fennelia	-		-		1	[10]
Fusarium	2	[29,35]	4	[8,16,32,44]	1	[11]
Geotrichum	2	[37,38]	2	[8,32]	1	[10]
Gibberella	-		-		1	[10]
Gliocladium	-		1	[16]	-	
Helminthosporium	-		1	[16]	1	[11]
Monilia	1	[35]	1	[14]	-	
Mycosphaerella	-		-		1	[10]
Nectria	-		-		1	[10]
Neosartorya[1]	-		1	[40]	-	
Ozonium	-		1	[16]	-	
Paecilomyces	-		4	[13,14,16,32]	1	[10]
Penicillium	17	[12,15,26-40]	16	[8,13,14,16,32,40-50]	8	[10,11,33,41,45,51-53]
Phoma	1	[37]	-		-	
Pleospora	-		-		1	[10]
Scopulariopsis	5	[15,30-32,38]	1	[32]	2	[51,53]
Stemphylium	-		1	[16]	-	
Trichoderma	-		2	[16,46]	2	[10,51]
Ulocladium	1	[29]	-		1	[10]
Wallemia	2	[26,29]	-		-	
Zygomycota						
Absidium	2	[12,35]	-		1	[53]
Actinomucor	-		-		1	[10]
Mortierella	1	[32]	1	[32]	1	[53]
Mucor	8	[12,15,26,28,29,32,37,38]	2	[16,44]	3	[10,11,53]
Rhizopus	3	[28,32,35]	3	[8,16,32]	3	[10,11,53]
Syncephalastrum	-		2	[13,32]	1	[10]
Thamnidium	1	[28]	-		-	
Mucorales genera	2	[31,34]	-		1	[51]

1) Names used in some references (synonyms used here according to taxonomy by Samson and Gams [56]): *A. amstelodami* , *A. chevalieri* , *A. glaucus*, *A. halophilicus*, *A. mangini*, *A. pseudoglaucus*, *A. repens, A. ruber* and *A. unguis* (*Eurotium*), *A. nidulans* (*Emericella), A. fischeri* (*Neosartorya*).
2) Names used in some references (synonyms used here according to taxonomy by Pitt [57]): *P. javanicum* (*Eupenicillium*).

Table 2: *Penicillium* and associated teleomorphic species isolated in studies of meat products. Isolated species, number of studies within it is isolated and references.

Species	Number of reporting studies					
	Fermented sausages		Dry-cured hams		Other meat products	
	No.	Ref.	No.	Ref.	No.	Ref.
Penicillium						
P. adametzii	1	[39]	-		-	
P. atramentosum	-		1	[41]	-	
P. aurantiogriseum	2	[28,36]	2	[13,49]	1	[10]
P. brevicompactum	6	[26,28,29,32,33,39]	6	[13,32,41,46,47,49]	4	[33,41,51,52]
P. camemberti[1]	3	[31,33,38]	1	[49]	2	[33,51]
P. canescens	-		1	[47]	-	
P. capsulatum	1	[26]	-		-	
P. cavernicola	-		1	[41]	-	
P. chrysogenum[1]	12	[15,26,28-34,36,38,39]	9	[8,13,32,41,42,44,47-49]	5	[10,33,41,51,52]
P. citreonigrum[2]	1	[29]	-		-	
P. citrinum[2]	3	[28,32,36]	6	[8,32,40,42,44,48]	4	[33,41,51,52]
P. commune[1]	10	[26,28-32,34,37-39]	6	[13,32,41,44,47,49]	3	[41,45,52]
P. concentricum	1	[33]	-		1	[33]
P. corylophilum	2	[29,33]	1	[8]	2	[33,52]
P. crustosum[1]	3	[26,35,36]	2	[40,41]	2	[41,52]
P. cyclopium[1,2]	3	[27,38,39]	6	[32,40,44-46,48]	2	[51,52]
P. daleae[2]	-		-		1	[33]
P. decumbens	-		-		1	[52]
P. discolor	-		-		1	[41]
P. digitatum	1	[33]	1	[8]	1	[33]
P. echinulatum	1	[37]	2	[13,41]	1	[33]
P. expansum	7	[28-30,32,37,38,40]	8	[8,13,32,40,41,44,46,49]	3	[33,51,52]
P. funiculosum	-		-		1	[33]
P. glabrum[2]	1	[33]	4	[32,44,45,47]	3	[33,51,52]
P. gladioli	1	[29]	1	[49]	-	
P. glandicola[1]	1	[36]	2	[8,40]	1	[52]
P. griseofulvum[1]	4	[30,32,36,39]	1	[49]	2	[33,51]
P. hirsutum[1]	1	[30]	-		2	[10,33]
P. hordei	-		-		1	[52]
P. implicatum	-		-		1	[52]
P. islandicum	1	[33]	-		2	[33,51]
P. italicum	1	[37]	-		2	[33,51]
P. janczewskii[2]	-		-		2	[10,33]
P. jensenii	-		2	[13,48]	-	
P. lanosum	2	[27,38]	1	[40]	1	[45]
P. nalgiovense	6	[26,29,30,33,37,40]	5	[40-42,47,49]	3	[33,41,52]
P. nordicum	1	[30]	1	[42]	-	
P. olsonii	4	[26,30,34,37]	-		-	
P. oxalicum	3	[26,30,37]	1	[13]	1	[10]
P. palitans	-		2	[40,41]	3	[41,51,52]
P. paxilii	1	[31]	-		-	
P. piceum	-		-		1	[52]
P. polonicum[1]	2	[26,31]	-		-	
P. purpurogenum	2	[33,38]	2	[8,32]	-	
P. purpurescens	1	[33]	-		1	[33]

Table 2: Continued.

Species	Number of reporting studies					
	Fermented sausages		Dry-cured hams		Other meat products	
	No.	Ref.	No.	Ref.	No.	Ref.
P. restrictum	-		1	[13]	-	
P. roqueforti	5	[28,29,31,32,38]	2	[32,42]	4	[33,41,51,52]
P. rugulosum	3	[28,33,38]	2	[13,32]	2	[33,52]
P. simplicissimum[3]	1	[32]	1	[46]	2	[33,51]
P. solitum	2	[26,37]	4	[40,41,47,49]	2	[10,41]
P. spinulosum	-		-		1	[51]
P. spathulatum (ined.)	1	[26]	-		-	
P. thomii	-		-		1	[52]
P. variabile	2	[29,33]	-		3	[10,33,51]
P. verrucosum[1]	7	[15,26,28,30,36,37,39]	4	[44,47-49]	-	
P. verrucosum var.[4]	2	[29,33]	-		1	[33]
P. viridicatum[1,2]	6	[26,30,31,37,38,40]	5	[13,32,40,46,49]	3	[10,51,52]
P. vulpinum[1]	1	[39]	-		-	
P. waksmanii	1	[36]	2	[47,49]	-	
Eupenicillium						
Eu.javanicum[2]	-		1	[32]	-	

1) Names used in some references (synonyms used here according to taxonomy by Frisvad and Samson [55]): *P. carneolutescens* (*P. polonicum*), *P. casei* (*P. verrucosum*), *P. caseicola*(*um*) (*P. camemberti*), *P. claviforme* (*P. vulpinum*), *P. cyaneofulvum* (*P. chrysogenum*), *P. granulatum* (*P. glandicola*), *P. lanosogriseum* (*P. commune*), *P. lanosoviride* (*P. commune*), *P. martensii* (*P. cyclopium*), *P. notatum* (*P. chrysogenum*), *P. ochraceum* (*P. viridicatum*), *P. olivinoviride* (*P. viridicatum*), *P. puberrulum* (*P. cyclopium*), *P. terrestre* (*P. crustosum*), *P. urticae* (*P. griseofulvum*), *P. verrucosum* var. *album* (*P. commune*), *P. verrucosum* var. *corymbiferum* (*P. hirsutum*).
2) Names used in some references (synonyms used here according to taxonomy by Pitt [57]): *P. albidum* (*P. daleae*), *P. dierckxii* (*P. citreonigrum*), *P. frequentans* (*P. glabrum*), *P. javanicum* (*Eu. javanicum*), *P. lanoso-coeruleum* (*P. cyclopium*), *P. nigricans* (*P. janczewskii*), *P. psittacinum* (*P. viridicatum*), *P. steckii* (*P. citrinum*).
3) Name used in some references (synonym mentioned by Frisvad [54]): *P. janthinellum* (*P. simplicissimum*).
4) Species varieties described by Samson et al. [58], which can be several species in section *Verrucosa* and *Viridicatum*

As a competent coloniser of meat products as well as being technologically and toxicologically suitable, *P. nalgiovense* has been used as mould starter culture within industrial mould-fermented sausage production since the 1970's [3,22]. *P. nordicum*, an ochratoxin A producing species, was a considerable part of the mycobiota in the studies where it was reported [30,42]. Its presence has most likely been underestimated as Larsen et al. 2001 [59] found that most of the closely related *P. verrucosum* strains isolated from cheese and meat had a secondary metabolite profile similar to *P. nordicum*. *P. nordicum* can like *P. nalgiovense* have white conidia and thus be indistinguishable from the starter culture [60]. Frequently reported *Aspergillus* species were *A. candidus*, *A. flavus*, *A. niger*, *A. ochraceus* and *A. versicolor*, while frequent *Eurotium* species were *E. repens* and *E. rubrum* (table 3). Among these, *E. repens* was in several cases the dominating species on dry-cured hams [13,44,48,49].

Table 3: *Aspergillus* and associated teleomorphic species isolated in studies of meat products. Isolated species, number of studies within it is isolated and references.

Species	Number of reporting studies					
	Fermented sausages		Dry-cured hams		Other meat products	
	No.	Ref.	No.	Ref.	No.	Ref.
Aspergillus						
A. caespitosus	1	[15]	-		-	
A. candidus	3	[26,29,32]	4	[16,44,49,50]	1	[51]
A. conicus	-		1	[50]	-	
A. flavipes	-		1	[14]	-	
A. flavus	3	[12,15,32]	5	[8,14,16,44,50]	3	[10,11,51]
A. fumigatus	-		5	[8,14,16,32,41]	2	[10,51]
A. gracilis	-		1	[50]	-	
A. melleus	-		-		1	[10]
A. microcysticus	1	[38]	-		-	
A. niger	3	[12,15,32]	5	[8,13,14,16,32]	2	[10,11]
A. ochraceus[1]	2	[30,36]	5	[8,14,16,44,50]	2	[10,51]
A. oryzae	-		-		1	[10]
A. parasiticus	1	[36]	1	[44]	1	[51]
A. penicillioides	1	[30]	3	[32,41,50]	-	
A. restrictus	-		3	[14,16,50]	-	
A. sydowii	1	[39]	2	[13,50]	2	[10,51]
A. terreus	1	[39]	1	[14]	1	[10]
A. versicolor	4	[15,29,30,35]	5	[13,14,16,32,50]	-	
A. viridinutans	-		1	[50]	-	
A. wentii	-		3	[14,16,32]	-	
Emericella						
E. aurantiobrunnea[1]	-		1	[50]	-	
E. nidulans[2]	-		1	[14]	-	
Eurotium						
E. amstelodami[2]	2	[30,32]	5	[8,32,41,48,50]	-	
E. chevalieri[2]	1	[32]	2	[8,32]	-	
E. halophilicum[2]	-		1	[8]	-	
E. herbariorum[2]	2	[29,35]	4	[13,14,32,41]	-	
E. pseudoglaucum[2]	-		2	[32,50]	-	
E. repens[2]	3	[26,32,38]	7	[13,32,44,46,48-50]	1	[51]
E. rubrum[2]	3	[26,30,32]	5	[13,32,47,49,50]	1	[51]
E. unguis[2]	1	[38]	-		-	
Neosartorya						
N. fischeri[2]	-		1	[40]	-	

1) Names used in some references (corrections according to Frisvad (pers. com.): *A. alutaceus* (*A. ochraceus*), *A. aurantio-bruneus* (*Em. aurantiobrunnea*).
2) Names used in some references (synonyms used here according to taxonomy by Samson and Gams [56]): *A. amstelodami* (*E. amstelodami*), *A. chevalieri* (*E. chevalieri*), *A. fischeri* (*N. fischeri*), *A. glaucus* (*E. herbariorum*), *A. halophilicus* (*E. halophilicum*), *A. manginii* (*E. herbariorum*), *A. nidulans* (*Em. nidulans*), *A. pseudoglaucus* (*E. pseudoglaucum*), *A. repens* (*E. repens*), *A. ruber* (*E. rubrum*), *A. unguis* (*E. unguis*).

Conidia, ascospores or mycelium fragments from the mycobiota of the surroundings and the air-spora inevitably contaminate the products, however a large part of these are not likely to grow on meat products [25]. Such sporadic contamination is most likely the cause for the

large number of genera and species in table 1-3 that were reported from only a few studies and in most occasions only in a small number within the studies. Conidia of *Cladosporium* and *Alternaria* are common in the atmosphere and occur in high concentrations in the air during summer and autumn [61], which explains their frequent occurrence on meat products. Skrinjar and Horvat-Skenderovic [39] reported *Cladosporium* to be the major contaminant of the fermented sausages in their study, otherwise *Cladosporium* and *Alternaria* have not been reported as dominating on fermented sausages or dry-cured hams.

Choice of sampling method, media and incubation conditions influences the diversity and composition of the mycobiota and thus has a large influence on the results [62]. Direct isolation of contaminating moulds reveals the dominating mycobiota, although slow growing and camouflaged moulds may not be detected. Methods using dilution and plating on agar reveal a larger part of slow-growing species, unless there is a large difference in the occurrence (1:10) between the species. The used medium and incubation temperature have a selective effect on the results. For example Lopez-Diaz et al. [34] used the medium dichloran rose bengal chloramphenicol agar (DRBC), a general-purpose medium for foods of high water activity (> 0.90), and obtained a large number of species belonging to Mucorales. As these had a rapid growth on the medium, isolation of more slow-growing species was difficult. Media selective for xerophilic, halophilic and/or proteinophilic species may be appropriate to assess the associated mycobiota of meat products, despite a smaller part of the diversity is assessed.

This summary reported filamentous fungi in studies of meat products of various geographical origins and provided genera and species frequently associated to meat products. The diversity was high and especially a large number of *Penicillium* species were reported. As mycotoxins are produced in a species-specific manner in food-associated *Penicillium*, correct identification of *Penicillium* species is extremely important [55]. The following manuscript covers a mycological survey of Danish meat products and the processing environments with a special focus on identification of *Penicillium* species.

Manuscript 1

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Mycobiota in the processing areas of two different meat products

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Abstract

Mould growth is not accepted on most types of North European meat products and is considered as both an economic and aesthetic problem for the producers. In order to determine the mycobiota in processing areas of fermented sausage and liver pâté, filamentous fungi were isolated from air, equipment and raw materials in the processing areas of two fermented sausage processing plants and two liver pâté processing plants. A total of 336 samples were examined. The diversity of filamentous fungi in the processing areas was high; at least 17 different genera were identified. The main isolated genera were identified as *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* and *Phoma*. Of these, *Penicillium* and *Eurotium* were the most important for contamination of fermented sausage, whereas *Penicillium* and *Cladosporium* were most important for liver pâté. *Cladosporium* was isolated more frequently in the processing plants examined in the autumn than in the spring. The seasonal variation indicates that outdoor air is an important source for this contamination. *Eurotium* was isolated frequently at one of the fermented sausage plants. *Penicillium* was isolated frequently at all four processing plants and was in addition found on moulded meat products. Sixteen *Penicillium* species were identified. The most frequently isolated were *P. brevicompactum* and the closely related *P. bialowiezense*, *P. solitum*, *P. palitans*, *P. fagi* and a new, not described species named *P.* "*milanense*" (ined.; Frisvad, 2007 personal com.). Isolation of a new species illustrates that the mycobiota in the processing areas of North European meat products has not yet been intensively investigated. Several mycotoxin producing species were isolated; the most prevalent were *P. brevicompactum*/*P. bialowiezense* and *P. palitans*. A screening for secondary metabolites showed that isolates of these species consistently produced mycophenolic acid and cyclopiazonic acid, respectively. Presence of these toxinogenic species in the processing areas implies a risk of mycotoxin contamination of the products if they are or has been subjected to mould growth. The ochratoxin A producing species *P. nordicum* and *P. verrucosum* were not isolated during the study. It was concluded that *Penicillium* species are the most important contaminants of the meat products because of their high prevalence in the production environment, their presence on meat products and their toxinogenic properties.
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Keywords: Fermented sausage; Filamentous fungi; Liver pâté; Meat products; Mycobiota; Processing areas

1. Introduction

Smoked, fermented sausage and liver pâté are among the commonly consumed meat products. The products are protected against microbial spoilage by preservation methods in combination with controlled atmosphere or vacuum packaging or by cold storage throughout the distribution chain. This is normally sufficient to prevent growth of filamentous fungi, which is traditionally not accepted on these types of meat products in North Europe. However, moulds periodically cause problems. On fermented sausages, moulds typically occur during the drying period if the pH decline is slow, if too little smoke is used or if wet surfaces occur during processing. When surface mould appears, it may be washed of, as it is the procedure at some producers (Singh and Dincho, 1994). If extensive growth has occurred it may lead to off-flavour development as well as contamination with mycotoxins, since some of the fungal

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species associated to meat products are able to produce mycotoxins (Frisvad and Thrane, 2002). In the case of liver pâté, mould growth typically occurs on the products during retail or after being purchased, which may result in consumer complaints. Mould growth on fermented sausage and liver pâté is therefore an important issue, as it may present both an economic, food safety and aesthetic problem for the producer.

The two products represent very different technological processes and product properties.

Smoked, fermented sausages are produced from minced meat and fat of pork or beef with added salt, sugar and starter culture (lactic acid bacteria and Staphylococci) for fermentation. The products are fermented, smoked and dried for 2–3 weeks at decreasing temperature (25–15°C) and decreasing relative humidity (down to approx. 92% RH). The finished products are often sliced, packed in controlled atmosphere or vacuum and stored cold. The fermented sausages are inherently preserved by low pH, low water activity and antimicrobial compounds from the smoke. Liver pâté is produced from minced liver and pork fat mixed with onions, wheat flour and spices. The pâté is baked, cooled and stored at low temperature. The indigenous biota of liver pâté is eliminated during baking, which also renders the product as an easily accessible substrate for contaminants after the heat treatment. Liver pâté is primarily preserved by storage at low temperature.

Only few studies have focused on the mycobiota in the processing areas of meat processing plants. Ismail et al. (1995) examined the surroundings in Egyptian abattoirs and Spotti et al. (1989), Andersen (1995) and Battilani et al. (2007) examined the air conidia of north Italian ham and fermented sausage production plants. Other authors have studied fungal contamination of meat products, but the examined products were primarily either mould-fermented or moulds were tolerated to some degree (rev. by Leistner and Eckardt, 1981; Grazia et al., 1986; Monte et al., 1986; Huerta et al., 1987; Mutti et al., 1988; Rojas et al., 1991; Nuñez et al., 1996; Peintner et al., 2000; Lopez-Diaz et al., 2001; Comi et al., 2004).

The objective of this study was to identify filamentous fungi present in the processing areas of fermented sausage and liver pâté. This information is used to determine the important fungi in terms of spoilage of the products and ability to produce mycotoxins. Special focus was on the identification of *Penicillium* species, as many species of this genus are mycotoxin producers. Differentiation of these species is important, since mycotoxins are produced in a species-specific manner in food-borne Penicillia (Frisvad and Samson, 2004).

2. Materials and methods

2.1. Sampling areas

A sampling of the processing areas, raw materials and products was done in four meat processing plants, two producing fermented sausage (plants A and B) and two producing liver pâté (plants C and D). Two of the samplings were in the spring 2005 (plants A and C), and two samplings were in the autumn 2005 (plants B and D). The processing areas were examined at 15–20 places pr. factory. This included raw materials sections, mincing/processing- and packaging areas in all plants, as well as the brining rooms, smoking cabinets and drying chambers in the fermented sausage plants and the baking areas and coolers in the liver pâté plants. Products were examined in case of visible mould spots.

2.2. Sampling methods

Equipment surfaces in the processing areas were examined by 4.5 cm Contact plates and by swabbing areas of approx. 200 cm^2 with swabs humidified with saline peptone water (0.9% NaCl and 0.1% Peptone). Air was examined by gravity sedimentation onto 9 cm Petri dishes for 2 h. Raw materials like spices, meat and fat and the finished products were examined by dilution in saline peptone water and subsequent plate spreading.

2.3. Sampling media and incubation conditions

All swab samples, air sedimentation samples, raw material and product samples were analysed for fungi on the two substrates DG18 (Dichloran 18% Glycerol agar) and CREAD (Creatine Sucrose Dichloran agar), while DG18 was used as substrate for Contact plates. Recipes were from Samson et al. (2002a). All plates were incubated at 20 °C for 5 days.

2.4. Isolation and identification of filamentous fungi

Fungal colonies with visible different appearance were isolated from each sample for further characterisation by 3-point inoculation on MEA (Blakeslee Malt Extract Autolysate agar), CYA (Czapek Yeast Autolysate agar) and YES (Yeast Extract Sucrose agar) agar plates (Samson et al., 2002a). After 5 days incubation at 20 °C, cultures from the same processing plant and with indistinguishable appearance on surface and reverse on all three substrates were considered similar. The different isolates were identified to genus level based on morphology of conidia and conidia-forming cells, type of conidia formation and colony morphology. *Penicillium* isolates were further inoculated on UNO (Urea Nitrate agar), CYAS (CYA with 5% NaCl) and CREA (Creatine Sucrose agar) agar and an Ehrlich test was made from colonies on CYA and YES (Frisvad and Samson, 2004). Morphology characteristics, growth physiology on the different media and the Ehrlich test were used for identification of the *Penicillium* to species level. All identifications were done according to Samson et al. (2002a) and Frisvad and Samson (2004).[1] To assure identification of closely related species, selected *Penicillium* strains were further examined for production of secondary metabolites by the use of the HPLC-based agar-plug method described by Smedsgaard (1997), see below. All the identified strains are maintained in the culture collection at the Danish Meat Research Institute, and selected *Penicillium* strains are maintained in the IBT culture collection at Center for Microbial Biotechnology, DTU Biosys.

[1] For species belonging to *Penicillium* subgenus *Penicillium*.

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Table 1
Number of samples examined from air, surfaces, raw materials and mouldy products at four meat processing plants producing fermented sausage (plants A and B) or liver pâté (plants C and D)

	Plant A	Plant B	Plant C	Plant D
	Fermented sausage	Fermented sausage	Liver pâté	Liver pâté
	Spring	Autumn	Spring	Autumn
Air	30	30	28	38
Surfaces	39	51	39	45
Raw materials	2	10	–	–
Mouldy products	18	2	4	–
Total	89	93	71	83

The processing plants were examined in the spring (plants A and C) or in the autumn (plants B and D).

2.5. Screening for secondary metabolites

The method is slightly modified after Smedsgaard (1997) with regard to solvents and HPLC conditions. The selected *Penicillium* strains were inoculated in 3 points on CYA and YES and incubated for 7 days in darkness at 25 °C. Three agar plugs from each culture were extracted ultrasonically for 60 min with 500 µl methanol/dichloromethane/ethyl acetate (1:2:3) containing 1% (v/v) formic acid. The dry extracts were dissolved in 300 µl methanol and filtrated. HPLC was performed on an Agilent HP1100 LC system with a 100 mm × 2 mm i. d., 3 µm, Luna C18II column (Phenomenex), coupled to an UV diode array detector and a fluorescence detector. A sample volume of 3 µl was loaded and the sample was eluted at a flow rate of 0.3 ml/min using a water–acetonitrile gradient system, buffered with 50 ppm triflouroacetic acid and with linear increase of acetonitrile from 15% to 100% in 20 min. An Agilent Chemstation was used for data collection. The metabolites were identified based on retention times, alkylphenone retention indexes and UV-spectra, which were compared with literature references and if available with standards.

3. Results and discussion

During sampling at the four meat processing plants a total of 336 samples were examined (Table 1). The level of fungal contamination varied between the individual meat processing plants (Table 2), probably influenced by the general hygiene, the buildings, the airflow, the outdoor environments and time of the year. The average contamination level among positive air samples was higher in the fermented sausage plants than in the liver pâté plants. The most contaminated samples were from smoke generators and smoking cabinets at the fermented sausage plants, and from coolers with high ventilation at the liver pâté plants.

Table 2
Percentage of samples infected with filamentous fungi from four meat processing plants and the average number of colony forming units in air or on surfaces (swab samples) among the positive samples

	Plant A	Plant B	Plant C	Plant D
	Fermented sausage	Fermented sausage	Liver pâté	Liver pâté
	Spring	Autumn	Spring	Autumn
Air	60% 13 cfu/2 h/plate	93% 16 cfu/2 h/plate	11% 1.7 cfu/2 h/plate	84% 4.5 cfu/2 h/plate
Surfaces	45% 13 cfu/cm^2	50% 1.6 cfu/cm^2	12% 0.6 cfu/cm^2	75% 15 cfu/cm^2

Table 3
Fungal genera isolated from four meat processing plants, quantified as the percentage of samples positive for the respective genus at each plant

Isolated genera	Plant A	Plant B	Plant C	Plant D
	Fermented sausage	Fermented sausage	Liver pâté	Liver pâté
	Spring	Autumn	Spring	Autumn
Acremonium		1%		
Alternaria				1%
Aspergillus		5%		2%
Aureobasidium	1%			
Botrytis		5%		1%
Cladosporium	4%	60%	6%	57%
cf. *Clonostachys*				1%
Epicoccum	3%	8%	1%	
Eurotium	1%	9%		
Fusarium		2%		2%
Paecilomyces		1%		
Penicillium	38%	22%	7%	13%
Phaeoacremonium				8%
Phoma		3%		11%
cf. *Pseudohansfordia*		1%		
cf. *Sporotrix*		1%		2%
Trichoderma			1%	

Approximately 300 fungal colonies were isolated, of which 128 isolates were considered to be different. Of these, 110 filamentous fungi were identified and belonged to 17 different genera (Table 3). The remaining strains were either yeast or could not be identified due to limited growth or conidium production.

The diversity of isolated fungi was relatively high, especially in the plants examined in the autumn. This probably reflects that fungal conidia are air-borne and are therefore easily spread. Important reservoirs can be humans, soil, dust, raw materials, drains, equipment surfaces and ventilation ducts (Scholte et al., 2002). Many of the genera were isolated rarely, only once or twice during the survey, which mean that they were most likely isolated by chance and were not representatives of a consistent house biota. Fungi present in more than five samples from one plant were considered as present in significant numbers.[2] From this criterion, the important genera were *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* and *Phoma*.

[2] When the total number of samples is taken into account, 5 positive samples are significantly different (95% level) from 0 positive samples according to a Fishers test.

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Cladosporium was present at all four plants and was isolated frequently from air and surfaces, but not from raw materials and products. *Cladosporium* is often found on meat and meat products and in the production surroundings (Leistner and Ayres, 1967; Racovita et al., 1969; Sutic et al., 1972; Jesenská, 1983; Grazia et al., 1986; Huerta et al., 1987; Skrinjar and Horvar-Skenderovic, 1989; Spotti et al., 1989; Rojas et al., 1991; Kaur et al., 1992; Andersen, 1995; Ismail et al., 1995; Nuñez et al., 1996; Ismail and Zaky, 1999; Mizáková et al., 2002). Many species of *Cladosporium* have a world-wide distribution and are commonly found in air and indoor environments (Samson et al., 2002a). The air conidia count has a seasonal variation and peaks in the late summer and autumn (Katial et al., 1997). In this survey we also found a higher incidence of *Cladosporium* in the plants examined in the autumn (57% and 60% of the samples) compared to in the spring (4% and 6% of the samples). Though *Cladosporium* occurred in high numbers, no products with this mould were observed. *Cladosporium* has in the past been reported as a problem on dry-cured ham and fermented sausages, where it causes black discoloration of the surfaces (Leistner and Ayres, 1967; Racovita et al., 1969). This indicates that the processing conditions of the investigated fermented products are unfavourable for growth of *Cladosporium*.

Penicillium was isolated frequently from both air and surfaces in the processing areas at all four processing plants and was the most frequent genus found on the meat products. This is in accordance with most previous studies. *Penicillium* species are common on various substrates including processed meats (Samson et al., 2002a). Among the *Penicillium* isolates 16 species were identified (Table 4). One of these is a new species named *P.* "*milanense*", which presently is under description (Frisvad, 2007 personal com.). This species has only been isolated from meat products and is closely related to *P. chrysogenum*. The finding of this new species illustrates, that the mycobiota in North European meat products has not been intensively investigated. As a note for this, the temperature used for incubations during sampling in this investigation was 20°C and the new species do not grow at 25°C, the normal temperature used for identification of fungi. This shows the importance of adapting the analysis method to the environment in question as also mentioned by Samson et al. (2002b).

Table 4
Penicillium species isolated from four meat processing plants, quantified as the percentage of samples positive for the respective species at each plant

Isolated *Penicillium* species	Plant A	Plant B	Plant C	Plant D
	Fermented sausage	Fermented sausage	Liver pâté	Liver pâté
	Spring	Autumn	Spring	Autumn
P. brevicompactum / P. bialowiezense	7%	4%	3%	4%
P. chrysogenum		1%		
P. commune	2%	2%		
P. corylophilum / cf. P. corylophilum	3%	1%		
P. cyclopium		1%		
P. decaturense		1%		
P. fagi	13%			
P. glabrum		1%	1%	1%
P. hordei		1%		
P. implicatum		1%		
P. milanense (ined.)	9%		4%	
P. palitans		10%		
P. purpurogenum		1%		
P. roqueforti	1%			
P. solitum	21%	4%	1%	8%
P. spinulosum		2%		

Five of the 16 *Penicillium* species occurred in significant numbers at one or more plants.

P. brevicompactum/P. bialowiezense[3] was found in all four plants. *P. brevicompactum* has been isolated from multiple different sources (nuts, fruits, cereals) and occurs commonly in soil and indoor environments (Samson et al., 2002a; Frisvad and Samson, 2004). It has been isolated from meat products in many studies, although never as the dominating species (Leistner and Ayres, 1967; Fiedler, 1973; Hadlok et al., 1975; Leistner and Eckardt, 1979; Grazia et al., 1986; Monte et al., 1986; Skrinjar and Horvar-Skenderovic, 1989; Andersen, 1995; Nunez et al., 1996; Peintner et al., 2000; Spotti et al., 2001). *P. solitum* was also found in all four plants. *P. solitum* is a contaminant on cheeses, meat products and air-dried fish (Samson et al., 2002a; Andersen, 1995; Peintner et al., 2000; Spotti et al., 2001; Kure et al., 2004; Tabuc et al., 2004; Papagianni et al., 2007). *P. palitans* was isolated frequently in plant B, but not in any of the other plants. *P. palitans* has previously been isolated from German and French meat products (Fiedler, 1973; Hadlok et al., 1975; Tabuc et al., 2004). Compared to *P. solitum* and *P. palitans*, *P. commune* has often been found in previous studies of meat products (Jircovsky and Galgóczy, 1966; Leistner and Ayres, 1967; Racovita et al., 1969; Skrinjar and Horvar-Skenderovic, 1989; Nunez et al., 1996; Spotti et al., 2001; Lopez-Diaz et al., 2001; Comi et al., 2004; Papagianni et al., 2007). *P. solitum*, *P. palitans* and *P. commune* are closely related species that are difficult to distinguish by morphological criteria (Lund, 1995). Here, they were identified based on differences in their metabolite profiles. The difficult identification is probably the cause why *P. solitum* and *P. palitans* relative rarely has been identified from meat products. In addition, *P. commune*, *P. palitans*, *P. solitum* and *P. cyclopium* have for a period been included in the morphologically based variety *P. verrucosum* var. *cyclopium*, which has also been reported from meat products (Leistner and Eckardt, 1979; Grazia et al., 1986; Skrinjar and Horvar-Skenderovic, 1989; ref. by Lücke, 1985). *P. milanense* (ined.) and *P. fagi* had a relatively high occurrence at plant A (fermented sausage). *P. fagi* was first isolated from fallen beech (Martínez and Ramírez, 1978) and is as *P. decaturense*, *P. roqueforti* and *P. spinulosum* typically found in soil, on wood or leaves. The source of these may be the wood used in the smoke generators.

Aspergillus was found with low occurrence in the environment of the two plants examined in the autumn (Table 3). This

[3] *P. brevicompactum* and *P. bialowiezense* are closely related species, which are rarely distinguished (Frisvad and Samson, 2004).

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genus is like *Penicillium* found on various substrates and is common in indoor air (Samson et al., 2002a). The occurrence is typical high in warmer climates, where *Aspergillus* occurs as a frequent mould on meat products (Sutic et al., 1972; Huerta et al., 1987; Mutti et al., 1988; Ismail and Zaky, 1999; Rojas et al., 1991; Kaur et al., 1992; Ismail et al., 1995; Nuñez et al., 1996; Comi et al., 2004; Wang et al., 2006).

Eurotium is the perfect state of the *Aspergillus glaucus* group. It was found relatively frequently (9%) from plant B, where it also occurred on a moulded sausage. *Eurotium* generally grows well on substrates with low water activity (Samson et al., 2002a) and it is often the dominating mould on dry-cured hams (Monte et al., 1986; Spotti et al., 1989; Nunez et al., 1996; Peintner et al., 2000; Spotti et al., 2001; Comi et al., 2004).

Phoma and *Phaeoacremonium* were isolated rather frequently from plant D (11% and 8% respectively), while *Botrytis* and *Epicoccum* both were found at plant B (5% and 8% respectively). *Phoma* is a plant pathogen and a saprophyte and is common in indoor air and in soil (Samson et al., 2002a). Species of *Phaeoacremonium* are known plant pathogens (Mostert et al., 2006). *Botrytis* is a plant pathogen, and is often found in humid, temperate climates on fruits and vegetables (Samson et al., 2002a). *Epicoccum* is a saprophyte and common in indoor air (Samson et al., 2002a). The high incidence of these plant pathogens and saprophytes in the meat processing plants examined during autumn could be due to high conidium counts of these fungi in the outdoor air at this time of the year. The air conidia count of *Epicoccum* do as *Cladosporium* have a peak in the late summer and autumn (Katial et al., 1997).

In the examination of filamentous fungi on moulded meat products *Eurotium*, *Trichoderma* and four *Penicillium* species were isolated. *Eurotium* occurred on a mouldy fermented sausage from plant B, the plant where it was also isolated most frequently (9%). Similarly, *P. solitum* and *P. fagi* were isolated from moulded fermented sausages from plant A, which agree with their high incidences (21% and 13% respectively) at this plant. Two other *Penicillium* species were also isolated from moulded fermented sausages from plant A. These were *P. corylophilum* and *P. roqueforti*, which were otherwise not found in the environment. *Trichoderma* was only isolated from liver pâté. The species causing mouldy products does therefore only partly correspond to the species occurring in the air and surfaces of the meat processing plants, at least in this survey. It was expected that more of the moulded products would have been infected with the typical meat-associated *Penicillium* species *P. commune*, *P. palitans* and *P. solitum*, as these are better adapted to the protein, lipid and salt contents of meat products than other species, but this was not the case.

Many *Penicillium* species can produce mycotoxins. At least half of the *Penicillium* species identified in this study are potentially able to produce toxic metabolites according to Frisvad and Thrane (2002). Some species of *Aspergillus* and *Eurotium* also produce toxic metabolites. Little is known about the toxinogenic potential of *Cladosporium* species (Frisvad and Thrane, 2002). Nuñez et al. (1996) tested one single strain of *Cladosporium* isolated from meat that showed no toxicity in 5 toxicity tests. In this study the most frequent toxinogenic *Penicillium* species were *P. brevicompactum*/*P. bialowiezense* (four plants) and *P. palitans* (one plant). Important toxic metabolites known to be produced by these species are the immunosuppressive drug mycophenolic acid (MPA) and the mycotoxin cyclopiazonic acid (CPA), respectively (Frisvad et al., 2004). In the screening for secondary metabolites, all tested strains of *P. brevicompactum*/*P. bialowiezense* ($n=4$) produced MPA as well as all tested strains of *P. palitans* ($n=5$) produced CPA (Table 5). The rest of their secondary metabolites do not have any known toxic properties. Fortunately, some of the meat-associated *Penicillium* that produce the most severe mycotoxins were not found in this study. This regards *P. nordicum* capable of producing the nephrotoxic and possibly carcinogenic mycotoxin ochratoxin A and *P. polonicum* capable of producing the tremorgenic mycotoxin verrucosidin (Frisvad and Samson, 2004; Frisvad et al., 2004). Finally, one of the most prevalent species in this study, *P. solitum*, is regarded as a non-toxinogenic species (Frisvad et al., 2004).

Table 5
Secondary metabolites produced by strains of *P. brevicompactum*/*P.bialowiezense*, *P. palitans* and *P. commune* grown for 7 days on CYA or YES at 25 °C

Species	No. of screened strains	Secondary metabolites detected (no. of strains)
P. brevicompactum/ *P. bialowiezense*	4	Mycophenolic acid (4), brevianamide A (1), mycochromenic acid (3), xanthoepocin (3), asperphenamat (3), quinolactacin (3), andrastin A (3).
P. palitans	5	Cyclopiazonic acid (5), palitantin (5), viridicatol (1), viridicatin (1), cyclopenol (1), dehydrocyclopeptin (1).
P. commune	2	Cyclopiazonic acid (2), viridicatol (1), viridicatin (1), cyclopenol (1), rugulovasin A (1), cyclopenin (1), cyclopeptin (1) dehydrocyclopeptin (1), cyclopolic acid (1).

4. Conclusion

The mycobiota in the processing plants was established momentarily and showed a high diversity of filamentous fungi mainly belonging to the genera *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* and *Phoma*. The important fungi with regard to spoilage of the fermented sausages were *Penicillium* and *Eurotium* species, as these were prevalent in the processing areas and were isolated from moulded sausages. For spoilage of liver pâté, the important fungi were *Penicillium* and especially in the autumn period *Cladosporium*. These were only isolated from the processing areas as very few moulded liver pâtés were found during this study. Further, continuous sampling of moulded products is needed to get a specific identification of the spoilage fungi as well as to assure focus on the problematic fungi in the individual processing areas. Many toxinogenic *Penicillium* species were isolated from the processing areas. Actively growing colonies on meat products therefore implies a risk of mycotoxins in the products. The high prevalence of *Penicillium* species in the processing areas and their presence on meat products does

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together with their toxinogenic properties make *Penicillium* species the most important filamentous fungi to be aware of in the production environment. As *Penicillium* was found in air and on surfaces in the processing areas, but not in any raw materials, the main contamination source is most likely outdoor air or an in-house living population rather than raw materials. The source of the prevalent food-associated *Penicillium* species as *P. brevicompactum*, *P. milanense* (ined.), *P. palitans* and *P. solitum* might be an in-house living population, but repeated examinations of the factories during the year are needed to determine this. Further knowledge of the contamination sources and survival of *Penicillium* species in the production environment would be beneficial in order to be able to control their presence in the processing areas.

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Part 2 Mycotoxins in meat products

Background

Contamination of meat products with toxinogenic filamentous fungi can be a potential source of mycotoxins in meat products [22]. The awareness of mycotoxin production by filamentous fungi increased in the 1960's after discovery of the toxicity of aflatoxin and a number of studies in the 1960-1970's have dealt with potential mycotoxin production by fungal isolates from meat products [15,33,50,63-68], reviewed by Leistner and Eckardt [22] and Leistner [69]. Since then the toxinogenic potential of filamentous fungi has been studied extensively and it has been realised that mycotoxin production is species-specific, at least in food-associated *Penicillium* [55]. Several of the *Penicillium* and *Aspergillus* species associated to meat products are toxinogenic (table 4), while the toxinogenicity of *Eurotium* spp. is still unclear [2,70]. The detrimental effects of the mycotoxins produced by fungi associated to meat products range from acute toxicity to chronic toxicity towards specific organs and in some cases carcinogenicity (table 5). Long-term exposure to low concentrations of chronic toxic mycotoxins is regarded as the major health hazard [71].

Growth of toxinogenic species on a meat product does not necessarily mean that they produce mycotoxins. Substrate, temperature, water activity and pH are some factors that can influence growth and alter mycotoxin production [72]. Thus, to evaluate the risk of mycotoxins in meat products it is necessary to examine empirically whether mycotoxins are produced on meat products, preferably at processing conditions. A number of studies have been conducted, where meat products have been inoculated with toxinogenic species and subsequently analysed for presence of mycotoxins. In some of these inoculation studies it was not possible to detect mycotoxins although products were inoculated with strains producing mycotoxins *in vitro* [44,65,66,73,74]; but limits of detection were not mentioned. Other inoculation studies have shown that mycotoxins can be produced by some species when growing on meat products (table 6), but in several cases the experimental conditions were far from what is encountered during production. Inoculation was for example on sliced, matured products and incubation conducted in closed containers. Spotti et al. [75,76] evaluated the ability of *P. verrucosum* to produce ochratoxin A (OTA) on fermented sausages under relatively realistic processing conditions. OTA was detected in the casing and in the peripheral part of the sausages, but not in the central part. The variation in OTA levels in the peripheral part of the sausages was found to follow the pattern of OTA levels in casings, although OTA was

detected later in the peripheral part of the sausages than in the casings. This showed that the tested strain of *P. verrucosum* was able to produce OTA under the conditions used for production of the fermented sausages.

Table 4: Mycotoxin production by filamentous fungi associated to meat products.
Toxinogenic species that have been isolated from meat products and known mycotoxins they are able to produce.

Species	Known mycotoxins[1]
A. flavus	Aflatoxin B_1, cyclopiazonic acid, 3-nitropropionic acid
A. niger	Ochratoxin A, fumonisin B_2
A. ochraceus	Ochratoxin A, penicillic acid, xanthomegnin, viomellein, vioxanthin
A. versicolor	Sterigmatocystin
P. aurantiogriseum	Penicillic acid, verrucosidin, terrestric acid, nephrotoxic glycopeptides
P. brevicompactum	Mycophenolic acid[2], botryodiploidin
P. chrysogenum	Secalonic acid, PR toxin, roquefortine C
P. citrinum	Citrinin
P. commune	Cyclopiazonic acid
P. corylophilum	-
P. crustosum	Terrestric acid, penitrems, roquefortine C
P. cyclopium[1]	Penicillic acid, xanthomegnins
P. expansum	Patulin, citrinin, chaetoglobosins, communesins, roquefortine C
P. glabrum	Citromycetin
P. griseofulvum	Patulin, griseofulvins, roquefortine C, cyclopiazonic acid
P. nalgiovense	-
P. nordicum	Ochratoxin A, viridic acid
P. olsonii	-
P. oxalicum	Secalonic acids, roquefortine C
P. palitans	Cyclopiazonic acid
P. roquefortii	Mycophenolic acid[2], PR toxin, roquefortine C
P. rugulosum	Rugulosin
P. solitum	-
P. variabile	Rugulosin
P. verrucosum	Ochratoxin A, citrinin
P. viridicatum	Penicillic acid, xanthoemegnins, viridic acid

1) Mycotoxins *sensu strictu*, based on Frisvad et al. [77] or Frisvad and Thrane [2].
2) Mycophenolic acid is not a mycotoxin *sensu strictu*, but an immunosuppressive compound [78].

Table 5: Important mycotoxins related to meat products.
Known toxic effects of mycotoxins related to meat products and values for provisional maximum tolerable daily intake (PMTDI).

Mycotoxin	Toxic effects[1]	PMTDI
Aflatoxin B_1	Carcinogenic (gr. 1), cytotoxic	Lowest possible [79]
Chaetoglobosin	Cytotoxic	
Citrinin	Cytotoxic, nephrotoxic	
Communesin	Cytotoxic	
Cyclopiazonic acid	Hepatotoxic, muscle and spleen toxicity	10 µg/kg bw [80]
Fumonisin B_2	Carcinogenic (gr. 2B), cytotoxic, hepatotoxic	2 µg/kg bw [81]
Mycophenolic acid[2]	Immunosuppressive	
Ochratoxin A	Carcinogenic (gr. 2B), hepato-, nephrotoxic, teratogenic	14 ng/kg bw[3] [82]
Patulin	Cytotoxic	0.4 µg/kg bw [83]
Penicillic acid	Cytotoxic	
PR toxin	RNA polymerase inhibitor	
Roquefortine C	Neurotoxic	
Sterigmatocystin	Carcinogenic (gr. 2B)	
Terrestric acid	Cardiotoxic	
Verrucosidin	Tremorgenic	
Viridic acid	Acute toxic	
Xanthomegnin	Hepatotoxic, nephrotoxic	

1) Toxicological effects are based on data reviewed by Hussein and Brasel [84], Bennett and Klich [71], Frisvad et al. [77] and evaluations of carcinogenic risks to humans by the International Agency for Research on Cancer (IARC) [85-87].
2) Mycophenolic acid is not a mycotoxin *sensu strictu* [78].
3) For comparison, this value was calculated from the provisional tolerable weekly intake (PTWI) of 0.1 µg/kg body weight [82] and thus the limit is for a accumulated weekly intake.

The depth to which mycotoxins contaminate meat depends on the ability of the mycelium of the producing species to penetrate into the meat and the ability of the mycotoxins to migrate in the particular product [22]. Diffusion of mycotoxins in meat products depends on the chemical properties of the mycotoxin as polarity and charge and the chemical and physical properties of the meat products as water and fat distribution and pH-value. Escher et al. [88] observed penetration of OTA approximately 0.5 cm into the meat of dry-cured ham, the layer where mycelium growth was also detected. While viable mycelium was not detected in the interior of the sausages by Spotti et al. [76], Grazia et al. [29] found viable mycelium deep within the interior of inoculated sausages. The contradicting result could be due to different strains, different experimental conditions or methodological. Anyway, Spotti et al. [75,76] detected OTA in the peripheral, inner part of sausages and thus the casings they used, natural

and artificial casings, did not prevent contamination of the meat with OTA. Incze and Frank [89] tested four different commercial casings and found that all were permeable to sterigmatocystin. Mycotoxins that are extruded from the mycelium are thus most likely to penetrate into the meat despite the use of casing or not.

Table 6: Mycotoxins produced on meat product after inoculation with filamentous fungi. Mycotoxins detected in meat products, inoculated species and types of products used for inoculation studies.

Mycotoxin	Species	Product	Reference
Aflatoxins[1]	*A. parasiticus*	Fermented sausages	[90-92]
	A. parasiticus	Slices of dry-cured ham	[14]
	A. flavus	Fermented sausages	[68,90,91,93]
Citrinin	*P. viridicatum*[2]	Slices of dry-cured ham	[94]
	P. citrinum	Slices of dry-cured ham	[95]
Cyclopiazonic acid	*P. viridicatum*[2]	Slices of dry-cured ham	[95]
Ochratoxin A	*P. verrucosum*	Fermented sausages	[75,76]
	A. ochraceus	Slices of dry-cured ham	[88]
Patulin	*P. expansum*	Fermented sausages	[96]
Sterigmatocystin	*A. versicolor*	Slices of dry-cured ham	[97]

1) Aflatoxin B1 or total aflatoxins (B_1,B_2,G_1,G_2) were reported.
2) The strains were incorrectly identified as citrinin and cyclopiazonic are not produced by *P. viridicatum* [77]

Studies reporting mycotoxin determination in commercial meat products are few, except in the case of OTA (table 7). The attention to OTA has largely been caused by the finding of a high contamination degree of barley used to feed pigs, deposition within kidney, liver and muscle tissue and thus also meat products [98]. Other possible indirect sources for presence of mycotoxins in meat products are ingredients as flour and spices if any are used. In one case it was likely that commercial fermented sausages were contaminated directly with OTA caused by growth of filamentous fungi on the surface [30]. In this survey of fermented sausages from several industrial and artisanal production plants, OTA was detected in the casings of 45 % of the products (n = 160). These sausages were described as densely covered with mould and the mycobiota contained the OTA-producing species *P. nordicum* and *P. verrucosum*, which were

the likely producers of the OTA. However, despite of detection of OTA at levels of 3 - 18 µg/kg on casings, they did not detect OTA in the interior meat (LOD =0.1 µg/kg). And after brushing and washing of the sausages, which were described as normal procedure, OTA was no longer detectable on the casings either. These results confirmed that OTA can and has been produced by the surface mould of fermented sausage during their production. But, although contradictory to previous results of Spotti et al [75,76], they also gave doubt of whether OTA penetrate into the meat.

Table 7: Examples of mycotoxins detected in commercial meat product.
Detected mycotoxins, type of products and incidence (positive / total no. of samples) and levels.

Mycotoxin	Product	Incidence	Detected levels	Reference
Aflatoxin B1	Luncheon meat	7 / 50	0.5 - 11 µg/kg	[10]
	Various meat products	7 / 135	2 - 150 µg/kg	[99]
Aflatoxin, total	Dry-cured meat products	8 / 40	2.8 - 47 µg/kg	[100]
Ochratoxin A	Fermented sausages	5 / 31	40 µg/kg	[39]
	Fermented sausages	28 / 56	0.01-0.27 µg/kg	[98]
	Fermented sausages	14 / 30	0.006 - 0.4 µg/kg	[101]
	Sausage casings[1]	72 / 160	3 – 18 µg/kg	[30]
	Dry-cured hams	32 / 60	0.2 – 2.0 µg/kg	[49]
	Dry-cured hams	35 / 42	0.2 – 2.3 µg/kg	[102]
	Dry-cured hams	5 / 10	0.1-7.3 µg/kg	[103]
	Liver pate	3/38	0.84-1.77 µg/kg	[104]

1) Casings of mould-fermented sausages, the interior meat did not contain detectable OTA (LOD = 0.1 µg/kg).

Presence of mycotoxins in meat products is not yet regulated by the European Union (EU) [105], but in some cases national regulation is more detailed. In Denmark where is for example a maximum tolerated level of 2 µg/kg aflatoxin B_1 that covers all foods not regulated by EU and thus also meat products [106]. The only European country with regulation of OTA in meat products is Italy with a maximum tolerated level of 1 µg/kg in pork and pork meat products [107]. However there is a maximum tolerated level of 25 µg/kg OTA in kidney from swine in Denmark [106]. The United States has a general regulation of aflatoxins (B_1,B_2,G_1,G_2) at 20 µg/kg that covers all foods [108]. Ingredients that are used for preparation of meat products may be regulated further.

Data on the occurrence of mycotoxins in meat products is necessary for assessment of exposure and is, together with toxicological data on the mycotoxins, a prerequisite for hazard assessment and the basis for establishment of mycotoxin limits and regulations [107]. Pioneer examinations of mycotoxins in meat products are thus of outmost importance to reveal whether they occur at all in meat products. Among the obstacles are the difficulties related to analytical determination of trace compounds in a complex matrix as meat products are.

Analytical methods for determination of mycotoxins have recently been reviewed by Turner et al. [109], Cigic and Prosen [110], Krska et al. [111] and Zöllner and Mayer-Helm [112]. The early methods applied for detection of mycotoxins in meat products were based on extraction with organic solvents and thin layer chromatography (TLC) on silica gel plates with visual, UV or fluorescence detection [88,94,96] and although TLC remain suitable for screening of a limited number of samples [95], the detection limits are to high for determination of trace levels. At present, methods for analytical determination of mycotoxins are mainly based on high performance liquid chromatography (HPLC) mostly using reversed-phase columns and organic eluents and coupled with UV, fluorescence (FLD) or mass spectrometry (MS) detection. UV detection requires complete separation from other UV-active compounds and is less sensitive than fluorescence and MS detection. FLD is usable for fluorophores and then has a good specificity and sensitivity. HPLC-FLD is the most frequently reported method for OTA determination in meat [98,101,103,104]. MS detection using electrospray ionisation (ESI) or atmospheric pressure ionisation (API) is usable for most mycotoxins and depends on an efficient and stable ionisation of the mycotoxin in the ion source [113]. Co-eluting interferences during LC-MS may lead to considerable ion suppression effects and thus a low and/or variable recovery of the mycotoxin [114]. A clean-up step is usually necessary when mycotoxins have been extracted from meat products in order to remove interfering compounds from the meat matrix that affect detection of the mycotoxin, but may also serve to up-concentrate trace amounts of mycotoxins. Fat, protein and degradation products hereof, salt and compounds from smoke, spices or other ingredients may be potential interferences. Liquid–liquid extraction has been used to remove excess fat from fatty samples or to clean-up acids and bases those polarity can be modified by changing the pH [115]. Otherwise solid phase extraction (SPE) with normal or reversed phase materials is the most generic method applicable for mycotoxins as well as anion or cation exchange

usable for acid- and base containing mycotoxins. Immunoaffinity columns (IAC) are highly specific, but are only available for the most common mycotoxins. IAC has been used a lot for clean-up of OTA from extracts of meat product [98,101,102]. Alternatives to HPLC methods have been developed for OTA. A simple method combining IAC with a fluorescence densitometer has been used to determine OTA in meat products [102]. Commercially available enzyme linked immunosorbent assay (ELISA) kits have been used as screening method for OTA determination in kidney and muscle tissue, but ELISA tended to slightly underestimate the OTA concentration when compared to a HPLC method [116].
As apparent, most methods applied for mycotoxin determination in meat products have been for OTA. However, the chemical diversity of mycotoxins that could potentially be produced in meat products is vast. Each group of compounds has different chemical and physical properties, as well as different meat products do. Thus the choice of method for extraction and separation of mycotoxins from meat must be selected and optimised for each individual mycotoxin and possibly also for different kinds of meat products. To obtain a reliable method, the method development must include validation of specificity, sensitivity, quantitative range, accuracy and reproducibility [117]. Development of analytical methods, especially for quantitative and validated methods, is a major task.

This summary revealed that many *Penicillium* and *Aspergillus* species frequently associated to meat products are toxinogenic and thus a large number of different mycotoxins can potentially be produced on meat products with mould growth. Observations regarding whether mycotoxins are actually produced on and penetrate into meat products are contradicting. The next manuscript describes a method for quantitative determination of mycophenolic acid in meat products. The method was applied for determination of MPA in fermented sausages, dry-cured hams and liver pâtés that had been inoculated with *P. brevicompactum* in order to determine if this species was able to produce mycophenolic acid on meat products during realistic processing conditions.

Manuscript 2

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Determination of mycophenolic acid in meat products using mixed mode reversed phase-anion exchange clean-up and liquid chromatography–high-resolution mass spectrometry

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ABSTRACT

A method for determination of mycophenolic acid (MPA) in dry-cured ham, fermented sausage and liver pâté is described. MPA was extracted from meat with bicarbonate–acetonitrile, further cleaned-up by mixed mode reversed phase-anion exchange and detected using a LC–MS system with electrospray ionisation-time-of-flight detection. The limit of detection was 4 μg/kg in sausage and 6 μg/kg in ham and pâté. The method was successfully used for quantification of MPA in dry-cured ham and liver pâté artificially inoculated with *Penicillium brevicompactum*. Levels ranged from 190 μg/kg in centre to 11 mg/kg in surface of ham and from 150 μg/kg in bottom to 14 mg/kg in surface of pâté.

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1. Introduction

Mycophenolic acid (MPA) is produced by filamentous fungi including *Penicillium brevicompactum*, *P. bialowiezense*, *P. carneum*, *P. roqueforti* [1] and *Byssochlamys nivea* [2]. MPA has immunosuppressive activity and is used in medicine at heart transplants to avoid organ rejection [3], with a maximum recommended dose for adult patients of 1.4 g/day [4]. MPA has often been described as a mycotoxin since it possesses animal toxicity, but because of relatively high lethal doses (700 and 2500 mg/kg for oral administration in rat and mouse, respectively [5]) it is not a mycotoxin sensu strictu according to the definition of Bennett [6]. Due to the immunosuppressive properties, prolonged exposure to MPA is suggested to be a health risk due to a higher susceptibility to bacterial and viral infections [3,7].

P. brevicompactum has been isolated from meat products in many studies [8–18]. As *P. brevicompactum* can produce MPA in numerous substrates as ginger [19], building materials [20] and most agar substrates [21,22], its presence on meat products raises the question if *P. brevicompactum* can produce MPA on meat products at processing conditions.

We have not been able to find any studies on quantitative analysis of meat products for MPA, and have only found one qualitative TLC-method (limit of detection (LOD) not reported) where MPA was not detected in artificially infected dry-cured hams [23]. Analytical methods for detection of MPA have been described for plasma, human skin, silage, food mixtures, cornflakes, bread and cheese [24–31]; LC–MS methods have been reviewed by Zöllner and Mayer-Helm [32]. Generally, studies of mycotoxins in meat products are based on tedious extraction procedures followed by solid-phase extraction (SPE) or immunoaffinity clean-up (not available for MPA) and/or directly analysed by TLC or HPLC with UV, fluorescence, MS or MS^n detection [33–38]. A weak acid like MPA (pK_a 4.7) has properties similar to ochratoxin A (OTA, pK_a 3.7). When extracting OTA from meat, both Chiavaro *et al.* [34] and Losito *et al.* [36] utilize that OTA is insoluble in water below its pK_a but very soluble at pH above its pK_a. Losito *et al.* [36] used sequential liquid–liquid partitions between polar and non-polar solvents by changing the sample pH and thereby the affinity of OTA for the respective solvents. They combined this relatively simple clean-up

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procedure directly with separation by HPLC–ESI-MS^n detection for quantification of OTA in pig tissues at the sub-ppb range.

We report on a method for determination of MPA in meat products based on basic extraction followed by mixed mode reversed phase-anion exchange and LC–MS. The method was applied for determination of MPA in dry-cured hams, fermented sausages and liver pâtés, that where artificially inoculated with a strain of *P. brevicompactum* isolated from dry-cured ham.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol were HPLC grade and obtained from Sigma–Aldrich (St. Louis, MO, USA). Pentane, formic acid, phosphoric acid, sodium bicarbonate and sodium acetate were analytical grade from Sigma–Aldrich. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). MPA of 98% purity was purchased from Sigma–Aldrich, stock solution of 10 mg/ml and further dilutions were prepared in acetonitrile.

2.2. Fungal cultures and meat raw materials

P. brevicompactum IBT 29319 was isolated from dry-cured ham and is available from the IBT culture collection at the Technical University of Denmark. Spore suspensions in water with 0.05% Tween 80 (Merck, Hohenbrunn, Germany) and 0.05% agar (Becton Dickinson, le Point de Claix, France) were made from cultures on malt extract agar (Blakeslee) supplemented with trace metal solution [39], incubated at 20 °C for 7 days. Salted raw hams and ready-made liver pâtés were delivered from two local manufacturers. Raw sausages were made in pilot plant facility at the Danish Meat Research Institute.

2.3. Inoculation, incubation and fractionation of meat samples

Raw hams and sausages were inoculated by immersion in a spore suspension (10^6 spores/ml).

Inoculated ($n = 4$) and control ($n = 2$) salted hams were dried in a climate chamber at the temperature and air humidity conditions normally applied for processing of dry-cured ham (confidential information). After drying (22 days) the rind was removed from each ham, a surface sample (1–2 cm layer) was cut from the remaining surface, and the rest of the ham was divided into a middle (2–3 cm layer) and centre fraction (3–4 cm). Inoculated ($n = 3$) and control ($n = 3$) sausages were dried at 24 °C and 95% relative humidity (RH) until fermentation was completed (48 h, pH 5.2), when temperature and humidity was lowered gradually to 15 °C and 92% RH. After a total drying time of 15 days, each sausage was divided into a surface (1 cm), middle (1 cm) and a centre fraction (2.5 cm). Liver pâtés were inoculated with 1 ml of a 10^5 spores/ml spore suspension spread onto the surface with a drigalsky spatula. Inoculated ($n = 5$) and control ($n = 3$) liver pâtés were closed with lid, and stored at 8 °C for 21 days. Each liver pate was divided into a surface (1–2 cm), middle (2–3 cm) and bottom (2–3 cm) fraction. All samples were stored at −20 °C until analysis.

2.4. Homogenisation, sampling and extraction

The whole meat samples (50–500 g) from each fraction of the products were homogenised in a blender and a sub-sample of 2.0 g was collected from at least 10 places in the homogenate. The samples were then shaken 5 min with 13 ml 3% (w/v) $NaHCO_3$ (pH 8.8), 7 ml acetonitrile and 10 ml pentane. After ultrasonication for 60 min and centrifugation 10 min at 3000 × *g*, the upper pentane phase was discarded and 10 ml of the bicarbonate–acetonitrile phase was removed and combined with 25 ml 0.2 M phosphoric acid. The samples were shaken to remove carbon dioxide, resulting in a pH of approximately 2.5.

2.5. Clean-up by mixed mode reversed phase-anion exchange

Oasis MAX cartridges (Vac RC Cartridge 60 mg 30 μm, Waters, Milford, MA, USA) were conditioned with 2 ml methanol and equilibrated with 2 ml H_2O. The samples (35 ml) were loaded at a flow of 1 ml/min in a vacuum manifold. The cartridges were then sequentially washed with 2 ml 50 mM sodium acetate pH 7–acetonitrile (9:1) and with 2 ml acetonitrile. The samples were eluted with 2 ml acetonitrile with 2% (v/v) formic acid, and the solvent was evaporated under N_2-flow or in a vacuum centrifuge. Samples were re-dissolved in 300 μl 35% acetonitrile in water, shaken on whirley mixer for 30 sec and ultrasonicated for 15 min. The samples were filtrated through 0.45 μm PTFE filters (National Scientific Company, Rockwood, TN, USA) and stored at −20 °C until LC–MS analysis.

2.6. LC–HRMS

LC–HRMS was performed on an Agilent 1100 LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode-array detector (scanning 200–700 nm), and a 50 mm × 2 mm i.d., 3 μm, Luna C18 II column (Phenomenex, Torrance, CA, USA), held at 40 °C. The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters) with a Z-spray electrospray ionisation (ESI) and a lock-mass probe [40]. A sample volume of 10 μl was injected and eluted at a flow rate of 0.3 ml/min using a water–acetonitrile gradient system starting from 25% acetonitrile that was increased linearly to 55% in 9 min and further to 100% in 2 min and with a holding time of 2 min. Water and acetonitrile was buffered with 20 mM formic acid. The MS-system was operated in negative ESI mode with a cone voltage at 30 V and capillary voltage at 2000 V.

2.7. Calibration, recovery and precision studies

Samples for spiked calibration curve were prepared by adding 100 μl of a MPA solution diluted to appropriate concentration to 2.0 g meat resulting in spiking levels of 100, 500 μg/kg, 1, 5 and 10 mg/kg. The spiked samples were kept at −20 °C over night until extraction. For determination of extraction and clean-up efficiency, MPA solution was added to 2.0 g meat, the bicarbonate–acetonitrile extract from 2.0 g meat or the cleaned-up extract from 2.0 g meat at spiking levels corresponding to 10 mg/kg in meat. All spiking studies were done in triplicate at three individual days. For recovery determination of pure MPA a series of concentrations from 0.66 to 66.6 μg/ml (5 levels) were prepared in 35% acetonitrile in water (9 replicates).

2.8. Data analysis

MassLynx 4.0 (Waters) was used for data collection and evaluation. At MS MPA was detected at a retention time of 6.97 ± 0.12 min, identified based on the molecular ion $[M-H]^-$ m/z 319.1182, and the m/z 319.09–319.15 extracted ion chromatogram was used for quantification. The ion $[M+Na-2H]^-$ m/z 341.1001 was used for confirmation. At high MPA concentrations UV detection at 216 nm was used to confirm the MS results. MPA was identified based on the retention time shift at UV compared to MS detection and the UV spectrum with the characteristic relative height of the absorption peaks at 216 (100%), 252 (24%) and 304 (12%) nm [40].

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2.9. Statistical analysis

A calibration curve was constructed for each matrix by linear regression to describe the relationship between spiked MPA concentration (X) and area of MPA (Y). A lack of fit test was used to determine whether the linear model was adequate to describe the data. When the intercept was not significantly different from zero in hypothesis test, the regression was redone without intercept. Statgraphics® Plus v. 4.0 (StatPoint Inc., Herndon, VA, USA) was used for linear regressions and lack of fit tests.

2.10. Method performance and validation

Instrument precision was determined from analysis of multiple MPA standard solutions, while day-to-day precision was determined at each spiking level (100 μg/kg to 10 mg/kg) for each matrix. Precision is expressed as % relative standard deviation (% RSD). Recovery was determined as the slope of a linear regression model describing the relationship between recovered MPA concentration (Y) and spiked MPA concentration (X) without any correction for matrix effect. LOD was set to the concentration corresponding to a signal-to-noise ratio of 3:1. Limit of quantification (LOQ) was set to the lowest concentration level for which a day-to-day precision lower than 25% RSD was obtained.

3. Results and discussion

3.1. The analytical method

Presence of MPA in meat samples may not be uniformly distributed. To account for this great care was taken to ensure homogenisation of the whole meat fraction sample and that the sampling of the 2.0 g subsample from the whole homogenate was done randomised.

Extraction was done at high pH (8.8) where MPA is unprotonated ($pK_a = 4.7$) and thereby has high affinity to a relatively polar solvent. Liquid–liquid partition with pentane was used to remove the very high contents of fat in some of the samples. A further clean-up was necessary to remove medium to polar interfering compounds. Reversed phase SPE was not sufficient and thus mixed mode reversed phase-anion exchange was used. The sample was loaded at low pH in polar solvent and MPA thereby attached by reversed phase action. When washed at neutral pH MPA becomes deprotonated and attaches to the anion exchange sites while non-acidic compounds are removed with acetonitrile. The HPLC method was optimised to a steep gradient with short run time resulting in a sharp MPA peak and good separation from major matrix peaks (Fig. 1). MS was run in negative mode as it predominantly afforded the $[M-H]^-$ ion and the $[M-2H+Na]^-$ ion in a ratio of approximately 5:1 and the in-source fragmentation to the $[M-H-HCOOH]^-$ ion was negligible. The ionisation efficiency was lower in positive mode and $[M+H]^+$ ions were repressed due to increased levels of $[M+Na]^+$ ions and formation of dimeric ions as $[2M+Na]^+$ (Fig. 2). Both positive and negative ionisation mode have been used successfully for MPA detection in previous studies, reviewed by Zöllner and Mayer-Helm [32]. The preference of negative mode ionisation in this study is therefore due to the specific combination of matrix and interface. The ion count was optimised at cone voltage and capillary voltage ranges from 20 to 30 and 2000 to 3000 V, respectively. Using a high-resolution MS instrument the detection was done in the narrow range m/z 319.12 ± 0.03 (319.09–319.15) giving a very low background noise compared to m/z 319.1 ± 0.5.

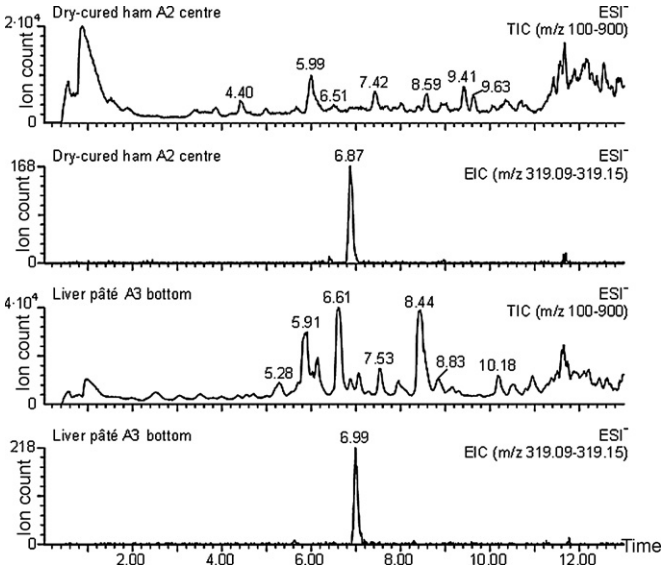

Fig. 1. LC–MS total ion count chromatogram and extracted ion chromatogram (m/z 319.09–319.15) of dry-cured ham centre fraction sample (190 μg/kg) and liver pâté bottom fraction sample (150 μg/kg).

3.2. Method performance

The MS apparent recovery of MPA in the different matrices varied from 29 ± 4% in dry-cured ham to 43 ± 1% in liver pate and 55 ± 11% in fermented sausages (Table 1), which are generally low recoveries. However, much higher recovery was obtained with quantification based on UV detection. To clarify this, the efficiency of extraction and clean-up was studied by spiking the extracts before and after the individual steps of the method and compare the recoveries. Both extraction and clean-up were satisfactorily efficient (>85%, Table 2) regardless the detection method used. Spiking into blank cleaned-up samples before LC–MS-DAD resulted in a recovery at UV detection of 111 ± 2%, which must be due to a minor degree of interferences. At MS detection spiking in the cleaned-up samples resulted in an apparent recovery of 66 ± 4% corresponding to a matrix effect of 34%. Ion suppression during ESI is therefore the major cause for the bias in recovery at MS detection. Rundberget and Wilkins [28] reported up to 40% reduction in peak area of Penitrem A when dissolved in food mixture extract compared to methanol–water mixture, which show that components present in the food matrix suppress the ionisation [28,30]. Recent published multi-analyte methods applied to various different matrices have shown that the matrix composition has a high influence on the degree of ion suppression [41,42].

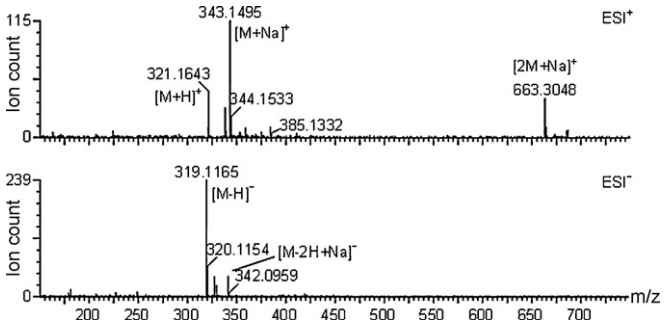

Fig. 2. Representative example of MPA ion spectrums at positive [47] and negative (Section 2.6) ionisation mode from fermented sausage spiked with 100 μg/kg.

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Table 1
Method performance parameters

Matrix	MS detection				UV detection			
	Recovery (%)[a]	RSD (%)[a]	LOD (μg/kg)	LOQ (μg/kg)	Recovery (%)[a]	RSD (%)[a]	LOD (μg/kg)	LOQ (μg/kg)
Dry-cured ham	29 ± 4[b]	20 ± 4	6	100	81 ± 4	4 ± 2	470	500
Fermented sausage	55 ± 11[b]	7 ± 4	4	100	86 ± 5	4 ± 2	230	500
Liver pâté	43 ± 1[b]	4 ± 2	6	100	93 ± 3	3 ± 0	470	500

Recovery, relative standard deviation (RSD), limit of detection (LOD) and limit of quantification (LOQ) for determination of MPA in dry-cured ham, fermented sausage and liver pâté with MS detection and UV detection.

[a] Recovery and RSD are average values ± 95% confidence intervals.

[b] Loss is mainly due to ion suppression.

Table 2
Efficiency of extraction, clean-up and detection method

Method step	Recovery (%)[a]	
	MS detection	UV detection
Extraction	100 ± 7	87 ± 5
MAX clean-up[b]	87 ± 9	94 ± 3
Detection method[c]	66 ± 4	111 ± 2
Total	53 ± 2	92 ± 3

Recovery of MPA calculated for the individual steps of the method. MPA was spiked at a level corresponding to 10 mg/kg in meat in triplicate into 2.0 g dry-cured ham, the bicarbonate–acetonitrile extract from 2.0 g dry-cured ham or the cleaned-up extract from 2.0 g dry-cured ham.

[a] Recovery (%) is average values ± 95% confidence interval.

[b] Mixed mode reverse phase-anion exchange clean-up.

[c] Spiking of blank cleaned-up samples before LC–MS-DAD.

Sulyok *et al.* [42] observed variation in matrix effect up to a RSD of 40% between rice varieties for some mycotoxins. Multi-analyte methods have also clearly shown that the degree of ion suppression is specific for the individual analyte [30,41–43]. Therefore, as Sulyok *et al.* [30] points out, an investigation of matrix effects (and extraction efficiencies) is required when a quantitative method is applied to a new analyte/matrix combination. We emphasize this statement, since the biological variation in matrix material appeared to be crucial in this study of three different meat products. Even the heterogeneity of the products may have an influence. The two spiking studies in dry-cured hams (Tables 1 and 2) were done using a mixture of fractions from blank hams and a centre fraction, respectively. The significantly different recoveries (presumable due to different water and salt contents) obtained in these spiking studies indicate how important it is to use exactly the same matrix material for spiked calibration curves as the samples to be analysed.

The day-to-day precision, expressed as % RSD, was determined from blank samples spiked at each of the concentration levels in the calibration curve and analysed at three individual days. With the exception of dry-cured ham the precision obtained was within the range accepted in trace compound analysis (up to 15–20% RSD) [44]. For comparison with the instrument precision, the RSD obtained from analysis of multiple MPA standard solutions was up to 6 ± 2% and 5 ± 3% with MS and UV detection, respectively. The day-to-day precision in dry-cured ham was generally low with MS detection (RSD = 20 ± 4%) indicating that the method robustness is influenced by the heterogeneity of the products and the high ion suppression effects. In order to get reliable, quantitative methods it is therefore important to develop efficient extraction and clean-up methods that can minimize the ion suppression when using MS as detection method. The degree of ion suppression is also most likely interface dependant [28,30]. The instrument sensitivity is influential since diluted sample or less sample volume can be injected at high-sensitivity MS instruments resulting in less matrix effect [45]. Use of a stable isotope-labelled standard of the analyte as internal standard may also be a way to account specifically for the ion suppression; however such is currently not available for MPA. As MPA is not a legally regulated compound and that the intended use of the method is for research purposes, we settled the acceptable precision limit to be a RSD below 25%.

LOD was based on a signal-to-noise ratio (S:N) of 3:1 (Table 1). Although the TOF instrument is not a high-sensitivity MS instrument, the LOD values at MS detection (4–6 μg/kg) were 60- to 80-fold lower than at UV detection (230–470 μg/kg). The better specificity and sensitivity of MS detection make the crucial difference when compared to UV-detection. For comparison, LOD of MPA in cheese matrices is in the range of 0.3 μg/kg reported by Kokkonen *et al.* [26] using extraction, liquid-liquid partition and a triple–quadrupole LC–MS/MS instrument, to 50–100 μg/kg reported by Zambonin *et al.* [31] using solid phase microextraction-HPLC. In a food mixture Rundberget and Wilkins [28] reported LODs of 70 and 10 μg/kg, respectively for a method using extraction, liquid–liquid partition and either LC–MS or LC–MS–MS on an ion-trap instrument. For determination of LOQ we used the definition that it should be the lowest level that can be accurately and precisely measured [46] and as described above the criterium was a RSD lower than 25%. With MS detection LOQ was set to

Table 3
Calibration parameters

Matrix	MS detection				UV detection			
	Slope[a]	Intercept[b]	Correlation coefficient	*n*	Slope[a]	Intercept[b]	Correlation coefficient	*n*
Dry -cured ham	0.096 ± 0.007	NS[c]	0.99	18	7.66 ± 0.24	NS[c]	1.00	15
Fermented sausage	0.209 ± 0.014	NS[c]	0.99	12	8.51 ± 0.18	NS[c]	1.00	10
Liver pâté	0.139 ± 0.002	NS[c]	1.00	18	8.83 ± 0.13	NS[c]	1.00	15

Slope and intercept from linear regression of MPA peak area as function of spiked MPA concentration in μg/kg, correlation coefficient and no. of samples (*n*) for spiked calibration curves in dry-cured ham, fermented sausage and liver pâté. Calibration ranges were 100 μg/kg to 10 mg/kg at MS detection and 500 μg/kg to 10 mg/kg at UV detection.

[a] Estimate ± 95% confidence interval.

[b] A hypothesis test was used to test if the intercept was equal to zero. When the intercept was not significantly different from zero, the regression was done without intercept.

[c] NS = not significant, 95% confidence level.

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Table 4
MPA in dry-cured hams

	MPA concentration (μg/kg)[a,b]		
	Centre fraction	Middle fraction	Surface fraction
Ham A1 (inoculated)	230	1100	4200
Ham A2 (inoculated)	190	2900	6000
Ham A3 (inoculated)	220	2100	4400
Ham A4 (inoculated)	200	3200	11,000
Ham E2 (control)	n.d.[c]	n.d.[c]	n.d.[c]
Ham E4 (control)	n.d.[c]	n.d.[c]	n.d.[c]

Levels of MPA in centre, middle and surface fractions of control hams and hams inoculated with *P. brevicompactum*.

[a] Method performance in dry-cured ham: RSD = 20 ± 4%, LOD = 6 μg/kg, LOQ = 100 μg/kg.

[b] $n = 1$.

[c] n.d. = not detected.

Table 5
MPA in liver pâtés

	MPA concentration (μg/kg)[a,b]		
	Bottom fraction	Middle fraction	Surface fraction
Pate A1 (inoculated)	n.d.[c]	4100	9400
Pate A2 (inoculated)	<LOQ	4500	11,000
Pate A3 (inoculated)	150	6100	14,000
Pate A4 (inoculated)	<LOQ	5200	8600
Pate A5 (inoculated)	<LOQ	3800	12,000
Pate B1 (control)	–	–	n.d.[c]
Pate B2 (control)	–	–	n.d.[c]
Pate B3 (control)	–	–	n.d.[c]

Levels of MPA in bottom, middle and surface fractions of control liver pâtés and liver pâtés inoculated with *P. brevicompactum*.

[a] Method performance in liver pâté: RSD = 4 ± 2%, LOD = 6 μg/kg, LOQ = 100 μg/kg.

[b] $n = 1$.

[c] n.d. = not detected.

100 μg/kg in all three matrices as this was the lowest concentration tested, while it was 500 μg/kg in all three matrices at UV detection.

3.3. Application to inoculated meat products

MS detection was used in the final method applied for inoculated meat products since the lowest LOQ values were obtained. Both extraction and clean-up losses as well as low recovery due to ion suppression effects were compensated for by making spiked calibration curves for each product by standard addition to the meat. Calibration curves (Table 3) in all three matrices were linear along a two-decade concentration interval as approved by a lack of fit test.

MPA was detected in dry-cured ham samples at levels from 190 μg/kg in centre fraction to 11 mg/kg in surface fraction (Table 4) and in liver pâté samples at 150 μg/kg in bottom fraction to 14 mg/kg in surface fraction (Table 5). It is therefore established here, that *P. brevicompactum* can produce MPA on meat products if the fungus is allowed to grow on the surface during processing and storage. MPA was found even in the centre and bottom fractions of dry-cured ham and liver pâté. However, in this study, the products were covered with a thick mould layer that in no case would have been accepted for human consumption. MPA was not detected in either the surface, middle or centre fractions of the three inoculated fermented sausages. It is possible that *P. brevicompactum* does not produce MPA in this product because of a relatively rapid pH decline and a shorter drying time compared to the dry-cured hams.

4. Conclusion

The method reported here enables the determination of MPA in three different meat products, dry-cured ham, fermented sausage and liver pâté, at levels down to 100 μg/kg. The method was well qualified for quantification of the MPA concentration levels produced by *P. brevicompactum* on meat products, and was precise enough to differentiate among the MPA levels in the different fractions of the meat products. With the method established here it was possible to conclude that MPA can be produced on some meat products by *P. brevicompactum*. Presence of actively growing colonies of *P. brevicompactum* on meat products should therefore be properly controlled. In general uncontrolled mould growth on meat products should be avoided, since the majority of food-related fungal species produce secondary metabolites that may be toxic or have other unbeneficial effects. Methods like this for determination of fungal secondary metabolites and mycotoxins are needed for various different matrices, at least for research purposes, to establish if the secondary metabolites may be produced or are actually present in foods and feeds.

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Part 3 Factors influencing mycotoxin production on meat products

Background

Several biological and environmental factors can influence the production of mycotoxins by filamentous fungi on meat products (illustrated in figure 4). The type of product and the processing conditions used, the species present and the degree of fungal growth have an impact on production of mycotoxins. The conditions used during processing and for preservation of meat products can directly or indirectly influence mycotoxin production by affecting fungal growth and development, by affecting the availability of precursors required for the biosynthesis or by affecting expression and activity of the enzymes involved in the biosynthetic pathway.

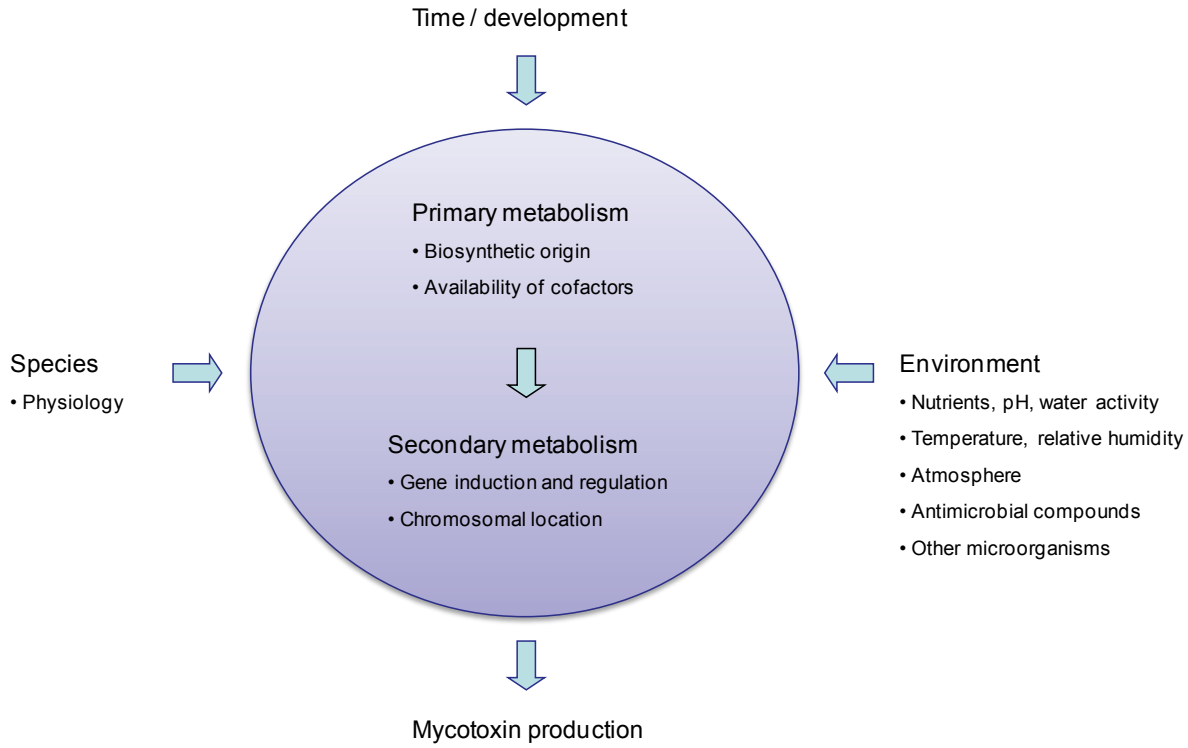

Figure 4: Factors that can influence production of mycotoxins.

Mycotoxins can be formed during fungal growth and the onset of mycotoxin production has been associated with developmental processes that also initiate sporulation [118,119]. Parts of a global coordination of vegetative growth, sporulation and secondary metabolism as well as pathogenicity have been revealed for certain *Aspergillus* spp. and for *P. chrysogenum* by mutation studies (reviewed by Calvo et al. [118], Yu and Keller [120], Brodhagen and Keller [121] and García-Rico et al. [122]). In very brief, so-called heterotrimeric G-proteins can be

activated by transmembrane receptors by external signals, which are largely unknown but may include hormones, nutrients and other environmental stimuli. When activated, the G-proteins can trigger the production of secondary messengers that in turn activate signalling pathways. On of these, the cAMP – protein kinase A signalling pathway, has been found to play a role in activation of vegetative growth and repression of conidiation and aflatoxin/sterigmatocystin production in *Aspergillus* spp. [118,120,121]. In this way the onset of mycotoxin production may be associated to development also in other species. The ability of a species to grow and differentiate on meat products is thus an important factor for mycotoxin production, which both depends on the physiology of the species present and the specific environment encountered on the product.

An important prerequisite for production of a mycotoxin, at the cellular level, is availability of precursors required for the biosynthesis [118]. Intermediates of the primary metabolism are the source of these precursors [120,123]. The backbone of polyketide mycotoxins is for example synthesised by condensation of short-chain carboxylic acids mainly from acetyl-CoA and malonyl-CoA, catalysed by iterative polyketide synthases in a similar manner to fatty acid synthesis [124,125]. The isoprene units of terpenoids are derived from condensation of acetyl-CoA and acetoacetyl-CoA as the first step in the mevalonate pathway. The nitrogenous parts of alkaloids are derived from amino acids as ornithine, lysine, tyrosine, trypthophan or phenylalanine. Non-ribosomal peptides are synthesised from amino acids by non-ribosomal peptide synthetases [123]. The pool of available precursors is expected to have an influence on the degree of mycotoxin production and may be an important factor especially during conditions with high production [120,126]. Medina et al. [127] did for example find that addition of phenylalanine to cultures of OTA-producing *Aspergillus* spp. enhanced production of OTA, which contains a phenylalanine-group in the molecule.

Another primary prerequisite for mycotoxin production is expression of the enzymes involved in the biosynthetic pathways and the actual production depends on a favourable regulation [118]. In recent years a number of studies have focused on gaining knowledge about the genetic basis for biosynthesis of secondary metabolites. This has included studies of genetic regulation and regulatory systems that connect secondary metabolism with development and signalling pathways triggered in response to environmental conditions. Several reviews with different angles of this topic have been published [118,120,121,125,126,128-132]. Genes encoding biosynthetic enzymes involved in secondary metabolite synthesis have been

recognised to be placed in gene clusters in the genome together with genes encoding proteins necessary for transport and regulation [133]. The biosynthetic genes have been found to be inducible rather than constitutively expressed and several studies have shown that the effect of environmental factors was asserted at the level of transcription [134-141]. In some cases a pathway-specific regulator has been identified. An example is the transcription factor AflR in the aflatoxin/sterigmatocystin gene clusters in *A. flavus*, *A. parasiticus* and *A. nidulans* [132]. In some other mycotoxin gene clusters as the fumonisin biosynthetic gene cluster in *Fusarium verticillioides*, there does not appear to be a specific regulator of the gene cluster [142]. However, several global regulators and regulatory systems have been found to influence transcription of mycotoxin gene clusters. The global regulator LaeA was shown to be necessary for *aflR* expression as well as production of several different secondary metabolites in *Aspergillus* species as *A. nidulans*, *A. fumigatus* and *A. terreus* and was itself regulated negatively by the cAMP – protein kinase A signalling pathway [143]. LaeA has also been recognised in *P. chrysogenum* and influenced biosynthesis of penicillin, but not roquefortine C [144]. Regulation of secondary metabolism in response to carbon catabolite repression, nitrogen metabolite repression and changes in pH mediated by the activity of the transcriptions factors CreA, AreA and PacC has partially been recognised for some species [120,131,132,134,145].

These studies of biosynthesis and regulation of mycotoxin production have mainly been restricted to species of medical, industrial or agricultural importance [126], but some of the general traits can be expected to be applicable also for the meat associated mycobiota. The current knowledge of environmental conditions that influence mycotoxin production by meat-associated *Penicillium* species is mostly based on physiological studies of single or few strains in laboratory substrates [21,72,146-149], supplemented by molecular biological studies of ochratoxin A-producing species [136,138,139]. Northolt and Bullerman [72] and Filtenborg et al. [25] have reviewed the effects of the most important environmental conditions that influence mycotoxin production in foods by food-related *Aspergillus* and *Penicillium* species. Among these are the water activity (a_w), temperature, atmosphere, pH and nutrients in the substrate.

For dry-cured meat products the a_w on the surface of products and the temperature used for ripening may be among the most important factors that influence production of mycotoxins. Mycotoxin production on meat products can be favoured under conditions of a_w and

temperature that are suboptimal for growth [22,139]. Schmidt-Heydt et al. [138,139] have for example in studies of *P. verrucosum* observed an optimum in OTA production and expression of the biosynthetic genes near the growth optimum, but also an additional minor optimum at an intermediate range where growth was restricted. Fermented, dry sausages and dry-cured meat products have been reported to be produced at temperatures typically between 15-26 ºC and 5 – 35 ºC respectively [4,14,41], while the a_w of meat products has been reported typically to be reduced from an initial value of approximately 0.95-0.96 to levels between 0.90-0.80 at the end of ripening [1,4,14,41,72]. The surface a_w of some dry-cured hams may reach a_w values of 0.77-0.79 at the end of ripening [13,47,150]. Compared to the ranges of a_w and temperature, where mycotoxin production has been observed by meat-associated species (table 8), the conditions of a_w and temperature encountered on meat products during ripening may not alone hinder mycotoxin production.

Table 8: Influence of water activity and temperature on mycotoxin production
Range of water activities (a_w) and range of temperatures where mycotoxin production has been observed for filamentous fungi associated to meat products.

Mycotoxin	Species	a_w range	Temperature range	Reference
Aflatoxin B1	*A. flavus*	≥ 0.84; ≥ 0.80[1]	12 - 35 ºC	[72,151]
Aflatoxin B1	*A. parasiticus*	≥ 0.84	12 - 35 ºC	[72]
Citrinin	*P. citrinum*	-	15 - 37 ºC	[119]
Cyclopiazonic acid	*P. commune*	≥ 0.90[2]	12 – 30 ºC	[148]
Ochratoxin A	*A. ochraceus*	≥ 0.87, 0.80[1]	12 - 35 ºC	[72,151]
Ochratoxin A	*A. niger* aggr.	≥ 0.90	10 - 35 ºC[2]	[152,153]
Ochratoxin A	*P. verrucosum*	≥ 0.85	2 – 34 ºC	[72,146]
Ochratoxin A	*P. nordicum*	-	15 - 30 ºC[2]	[136]
Patulin	*P. expansum*	≥ 0.95	0 - 28 ºC	[72,119]
Verrucosidin	*P. polonicum*	≥ 0.95[2]	12-30 ºC	[147]

1) Obtained on substrate equilibrated to the indicated a_w by adsorption of water.
2) The value(s) shown here were the range investigated and thus the actual range may be wider.

A detailed study of ranges of a_w and temperature that allowed mycotoxin production by strains of selected *Aspergillus* and *Penicillium* species has been reported by Northolt and Bullerman [72]. The ranges of a_w and temperature that allowed mycotoxin production were found to be dependant on the substrate and were species-dependant, although the temperature-range varied within strains. The minimum temperatures for growth as well as mycotoxin

production were found generally to be lower for *Penicillium* spp. than for *Aspergillus* spp. In some cases mycotoxins were produced almost during the whole range of a_w and temperature that allowed growth, while in other cases the ranges where mycotoxin was produced were much more restricted. Aflatoxin B_1 was for example produced by *A. flavus* and *A. parasiticus* at a_w values of 0.85 and temperatures down to 10 ºC, which were close to the minimum a_w and temperature for growth, whereas patulin production by *P. expansum* was confined to high a_w values above 0.97 [72]. The compound used to adjust a_w in laboratory media may influence the results. The OTA production by *P. verrucosum* was for example lower when NaCl was used compared to glycerol, at least at high a_w values [138].

In the production of fermented sausages pH is lowered from the initial value of approximately 5.6 to about 4.8 (4.5-5.0) during the fermentation [4], which may have an influence on the mycotoxin production of some species. Culture medium with pH 4 has been shown to support OTA production by both *P. verrucosum* and *P. nordicum*. While there for *P. nordicum* was observed a decrease in OTA production along with a reduction from pH 5 to pH 4, there was a minor increase for *P. verrucosum* [136,139]. OTA production by *A. niger* aggr. spp. has been shown to be supported in pH range from 2-10 in culture media [154]. Aflatoxin B_1 production by *A. parasiticus* has been shown to be enhanced at pH 4 compared to neutral pH [134,155].

The substrate can have a large effect on mycotoxin production and it may thus be relevant to consider the influence during changes in the product formulation of meat products. Detailed investigations have been conducted for aflatoxin production by *Aspergillus* spp. and have been reviewed by Luchese and Harrigan [20] and Cary and Calvo [129]. Carbon sources that are readily oxidized as glucose, maltose, fructose, sucrose, ribose and xylose were reported to be excellent for aflatoxin production, while lactose, galactose, rhamnose, arabinose, cellubiose, soluble starch, oleic acid and most amino acids were reported to be poor or non-supporting carbon sources for aflatoxin production. High sugar concentrations generally lead to high aflatoxin levels produced [20]. Nitrogen sources as ammonium, yeast extract and peptone were reported to result in higher aflatoxin production than nitrate [20]. Cyclopiazonic acid production by *P. commune* and verrucosidin production by *P. polonicum* were also enhanced by presence of glucose in the substrate [21,147]. Patulin production by *P. expansum* was supported on medium with starch and glucose [149]. In contrast, OTA production by *P. nordicum* has been reported to be lower on glucose and nitrate compared to glycerol and

ammonium [156], as well as OTA was detected at fairly higher levels on a cheese analogue compared to a substrate with high sucrose concentration [157].
Some minor constituents as for example preservatives and compounds from spices, smoke or products of other microorganisms may have a stimulating or inhibiting effect on growth and mycotoxin production [158]. Low concentrations of some organic acids as acetic acid, propionic acid and butyric acid have been reported to inhibit aflatoxin production by *Aspergillus* spp. [159,160], while low concentrations of lactic acid and sorbic acid have been reported to stimulate aflatoxin production at conditions where carbon sources as glucose were present [20,161-163]. Schmidt-Heydt et al. [138] observed that low concentrations of calcium propionate and potassium sorbate stimulated OTA production and expression of the gene encoding the polyketide synthase involved in OTA biosynthesis by *P. verrucosum*, especially at low water activities. A large number of studies of the inhibitory effects of spices or compounds from spices on aflatoxin production have been reviewed by Zaika and Buchanan [158] and included studies of for example cinnamon, clove and pepper. Smoking of fermented sausages inoculated with *A. flavus* was shown to delay the aflatoxin production [93].
The presence of other microorganism may as well influence mycotoxin production. Most studies have been conducted for monocultures, however Incze and Frank [63] tested experimentally whether aflatoxin production was influenced by mixed cultures of aflatoxin producers and several other *Penicillium* species present on meat products. At high a_w and temperature aflatoxin was produced both in monocultures and mixed cultures, but when the a_w or the temperature was reduced, aflatoxin was not produced in the mixed cultures. Contrary there have been examples of stimulation of aflatoxin production by presence of lactic acid bacteria [155].

As apparent from this summary of literature, there are complex relations among factors that affect mycotoxin production and it may be difficult to deduce how mycotoxin production is affected on a meat product. However a throughout knowledge of the factors that influence growth and mycotoxin production of the biota associated to meat products would be useful for predicting how the conditions used during processing and for preservation of meat products influence the potential mycotoxin production by contaminating moulds. The next manuscript describes a study conducted in order to improve the knowledge of the cellular mechanisms that influence fumonisin B_2 production by *A. niger*.

Manuscript 3

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Proteome analysis of *Aspergillus niger*: Lactate added in starch-containing medium can increase production of the mycotoxin fumonisin B_2 by modifying acetyl-CoA metabolism

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Abstract

Background: *Aspergillus niger* is a filamentous fungus found in the environment, on foods and feeds and is used as host for production of organic acids, enzymes and proteins. The mycotoxin fumonisin B_2 was recently found to be produced by *A. niger* and hence very little is known about production and regulation of this metabolite. Proteome analysis was used with the purpose to reveal how fumonisin B_2 production by *A. niger* is influenced by starch and lactate in the medium.

Results: Fumonisin B_2 production by *A. niger* was significantly increased when lactate and starch were combined in the medium. Production of a few other *A. niger* secondary metabolites was affected similarly by lactate and starch (fumonisin B_4, orlandin, desmethylkotanin and pyranonigrin A), while production of others was not (ochratoxin A, ochratoxin alpha, malformin A, malformin C, kotanin, aurasperone B and tensidol B). The proteome of *A. niger* was clearly different during growth on media containing 3% starch, 3% starch + 3% lactate or 3% lactate. The identity of 59 spots was obtained, mainly those showing higher or lower expression levels on medium with starch and lactate. Many of them were enzymes in primary metabolism and other processes that affect the intracellular level of acetyl-CoA or NADPH. This included enzymes in the pentose phosphate pathway, pyruvate metabolism, the tricarboxylic acid cycle, ammonium assimilation, fatty acid biosynthesis and oxidative stress protection.

Conclusions: Lactate added in a medium containing nitrate and starch can increase fumonisin B_2 production by *A. niger* as well as production of some other secondary metabolites. Changes in the balance of intracellular metabolites towards a higher level of carbon passing through acetyl-CoA and a high capacity to regenerate NADPH during growth on medium with starch and lactate were found to be the likely cause of this effect. The results lead to the hypothesis that fumonisin production by *A. niger* is regulated by acetyl-CoA.

Background

Aspergillus niger is a versatile filamentous fungus found in the environment all over the world in soil and on decaying plant material and it has been reported to grow on a large number of foods and feeds [1]. At the same time it is a popular production host for industrial fermentations and it is used for production of both organic acids and for indigenous and heterologous enzymes and proteins [2-4]. However, *A. niger* produces various secondary metabolites, and among those also the important mycotoxins fumonisin B_2 (FB_2) and ochratoxin A (OTA) [5,6]. Due to the ubiquity of *A. niger*, its production of secondary metabolites is important both from a biotechnological and a food-safety viewpoint.

Secondary metabolites are small molecules that are not directly involved in metabolism and growth. Both plants and fungi are known for producing a large number of chemically diverse secondary metabolites. While the role of some of these metabolites makes sense biologically as inferring an advantage to the producer, e.g. antibiotics, virulence factors, siderophores and pigments, the benefit of others is less obvious or unknown. The general belief is that the secondary metabolites must contribute to the survival of the producer in its environment where it competes with other organisms [7]. Whereas the ability to produce individual secondary metabolites is species-specific, the actual production of secondary metabolites has, in broad terms, been reported to be affected by the developmental stage of the fungus (i.e. conidiation) and intrinsic and extrinsic factors of the environment as substrate (composition, pH, water activity), temperature, light and oxygen availability [8-12].

Fumonisins are a group of secondary metabolites with a highly reduced polyketide-derived structure consisting of a hydrocarbon backbone with an amino group in one end, some methyl groups and two ester-bound side groups consisting of tricarballylic acid moieties. The fumonisin B-series group contains up to three hydroxyl groups and the degree of hydroxylation gives rise to the designations B_1-B_4[13,14]. These are classified as mycotoxins as they have been shown to be cytotoxic and carcinogenic [14,15] and fumonisins have been suspected to be involved in oesophageal cancer in South Africa and China [16-19]. Fumonisin production in *Fusarium* spp. has been known since the 1980's [20], while the ability of *A. niger* to produce FB_2 was just discovered in 2007 based on indications from the genome sequencing projects of *A. niger* ATCC 1015 and CBS 513.88 [6,21,22]. The fumonisin biosynthesis pathway and the gene cluster are partly characterized in *F. verticillioides* and include a polyketide synthase (Fum1), fatty acyl-CoA synthetases (Fum10, Fum16), an aminotransferase (Fum8), a short chain dehydrogenase/reductase (Fum13), cytochome P450 monooxygenases (Fum6, Fum12 and/or Fum15) and a dioxygenase (Fum9) [23]. The expected fumonisin biosynthesis gene cluster in the *A. niger* CBS 513.88 genome contains 14 open reading frames of which a number has similarity to the fumonisin biosynthesis cluster genes in *F. verticillioides* [22]. Although the knowledge of the biosynthesis pathway is incomplete, the expected precursors and cofactors required for production of fumonisins are acetyl-CoA, malonyl-CoA, methionine, alanine, 2-ketoglutarate, O_2 and NADPH [13].

Due to the late discovery of FB_2 production in *A. niger*, its ability to produce this metabolite has only been the subject of a few studies. *A. niger* was shown to be a relatively consistent producer of FB_2 on media such as Czapek yeast autolysate agar (CYA) with 5% NaCl [6,24], yet it was noted that the media that support FB_2 production in *A. niger* were different from those who were supportive in *F. verticillioides* [6].

To evaluate the potential risk of mycotoxin production in foods and feeds, we explored the influence of substrate on FB_2 production by *A. niger*. During our screening of food-related carbon sources as glucose, sucrose, lactate, starch and fat we found that lactate, when added to a medium containing starch, could synergistically increase the FB_2 production compared to either starch or lactate alone. To reveal a biological explanation for this interesting observation, we combined growth physiology studies including measurement of several secondary metabolites with a proteome study.

Proteome studies give information about the capability for metabolic flow in the cell, for maintenance of the cell and for anabolic and catabolic processes. The proteome constitutes the cellular machinery, is energetically expensive to maintain and has a crucial influence on the fitness of the fungus. Protein synthesis and degradation are thus carefully regulated at multiple levels. The use of proteome analysis within studies of filamentous fungi has attracted increasing interest in these years and has recently been reviewed by Carberry and Doyle [25], Kim et al. [26,27] and Andersen and Nielsen [28]. The emergence of fungal genome sequences combined with continuously improved mass spectrometry technologies will further show proteomics as useful for studies in fungal biology.

We report on a 2D gel based proteome study conducted to relate differences in protein levels with differences in secondary metabolites especially FB_2 production, and with the aim of elaborating on the reasons for an increased FB_2 production on medium containing starch in combination with lactate.

Results and discussion

Growth and secondary metabolite production

For these experiments we used a wildtype *A. niger* isolate (*A. niger* IBT 28144) that is able to carry out normal metabolism and synthesis essential for growth and survival in a natural habitat. Additionally it was able to produce both of the two mycotoxins FB_2 and OTA. With the aim to explore factors that influence secondary metabolism, especially FB_2 biosynthesis, we used this isolate, grown on the surface of a solid medium and with a moderately rich substrate containing amino acids, nitrate, vitamins, minerals, trace metals and the polysaccharide starch (Czapek Yeast Autolysate agar with saccharose replaced by starch and/or other carbon sources). *A. niger* IBT 28144 grew vigorously under these conditions (Figure 1). Mycelium was observed 20 hours after inoculation and biomass accumulated within 70 hours. Aerial hyphae, the first sign of onset of conidiation, were observed already after 24 hours.

To measure the production of secondary metabolites we used a modified version of a micro-scale extraction procedure [29] that is suitable for detection of a wide array of metabolites. Using plug sampling, the amount of secondary metabolites was determined per surface area of the culture including both metabolites within the cells and metabolites diffusing into the medium. Using this method we detected the following metabolites produced by *A. niger* on starch-containing medium; fumonisin B_2, fumonisin B_4, ochratoxin A, ochratoxin alpha, malformin A, malformin C, orlandin, desmethylkotanin, kotanin, aurasperone B, pyranonigrin A and tensidol B.

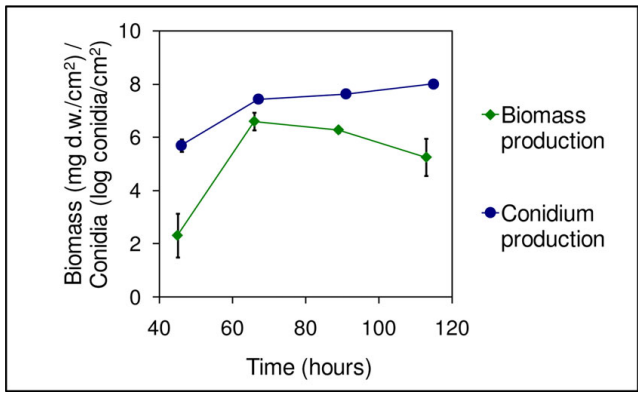


Figure 1
Growth and conidium production. Growth measured as biomass production (mg dry weigth/cm^2) and conidium production (log conidia/cm^2) by *A. niger* IBT 28144 on medium containing 3% starch. Average values ± standard deviations (n = 3-6).

Presence of lactate, which may be encountered in environments with fermenting microorganisms and especially in fermented food products, was found to increase FB_2 production considerably when supplied in tandem with starch. The FB_2 levels detected on media with 3% starch plus 3% lactate were 2-3 times higher than the levels on 3% starch. The differences were significant (95% confidence) at the samplings 66, 92 and 118 hours after inoculation (Figure 2). The stimulating effect of lactate on FB_2 production seemed to be proportional to the concentration of lactate as 3% starch plus 1.5% lactate resulted in levels intermediate of those containing 3% starch and either no lactate or 3% lactate. Fumonisin B_4, orlandin, desmethylkotanin and pyranonigrin A were regulated like FB_2 but only during the later growth phase (Figure 3). Especially the level of the polyketide orlandin was increased synergistically by the combination of starch and lactate. Orlandin, desmethylkotanin and kotanin have very similar polyketide structures and are expected to be part of the same biosynthesis pathway [30], but kotanin was not influenced in the same way as orlandin and desmethylkotanin by presence of starch and lactate. The differential influence of starch and lactate on production of the 12 measured metabolites indicates that secondary metabolism of *A. niger* is not restricted to a common regulation under these conditions. Presence of starch was important for both the growth and the production of secondary metabolites; all were lower on 3% lactate compared to 3% starch with the exception of the ochratoxins that were produced at similar amounts on lactate and starch.

We considered whether the effect of lactate in combination with starch could be due to a specific induction of secondary metabolite synthesis by lactate and if this

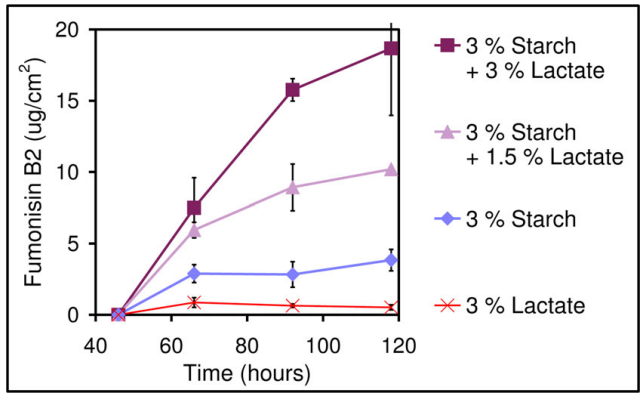


Figure 2
Fumonisin B_2 production. Levels of fumonisin B_2 (μg/cm^2) produced by *A. niger* IBT 28144 on media containing 3% lactate, 3 % starch, 3 % starch + 1.5 % lactate and 3 % starch + 3 % lactate. Average values ± standard deviations (n = 3-18).

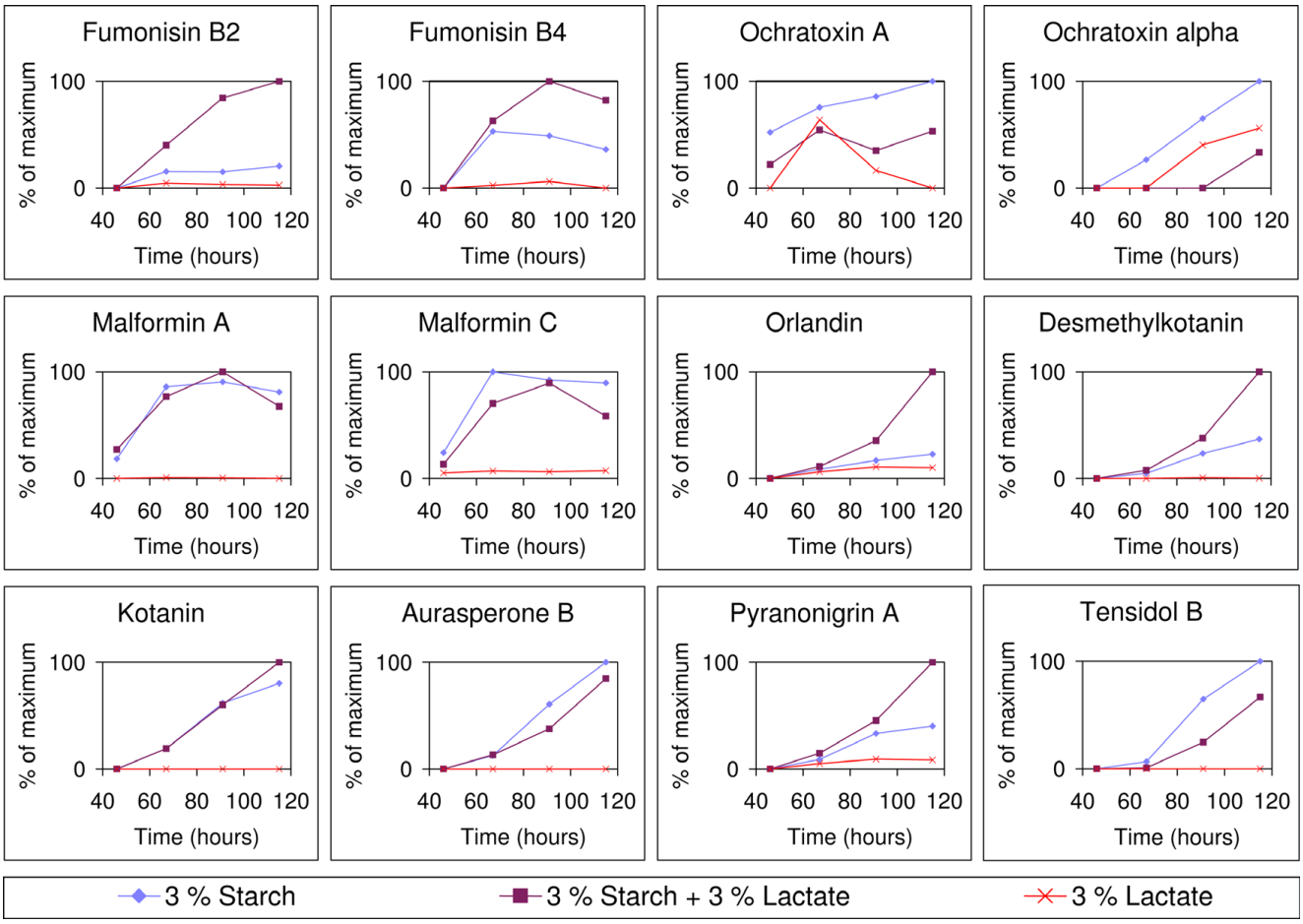


Figure 3
Secondary metabolite production. Production of selected secondary metabolites produced by *A. niger* IBT 28144 on media containing 3% starch, 3% starch + 3% lactate and 3% lactate. Data based on average peak area per cm^2 (n = 3) calculated as percentage of maximum value obtained for each metabolite.

could constitute some kind of antimicrobial defence. However we found that pyruvate, a product of L-lactate degradation (eq. 1 and 2), had a similar effect (Table 1), which makes an effect of lactate itself unlikely and to a higher degree pointing to an effect of lactate degradation.

While it is well known that starch is degraded by extracellular enzymes to maltose and glucose, transported into the cell and then entering glycolysis, we may assume that lactate is transported into the cell by a lactate transporter and mainly metabolized further to pyruvate by a L-lactate dehydrogenase (EC 1.1.1.27) or a L-lactate dehydrogenase (cytochrome) (EC 1.1.2.3), both are predicted to be present in the genome. While the medium with 3% starch + 3% lactate contains approximately the double amount of added carbon source (the yeast extract contains carbon sources as well) compared to the media with 3% starch or 3% lactate alone, it is possible that this is partly counteracted by carbon catabolite repression of the lactate transporter, as the activity of the lactate transporter in yeast, Jen1p, is inversely related to the

Table 1: Fumonisin B_2 production on different carbon sources

Supplemented carbon source	Fumonisin B_2[1,2] (μg/cm^2)	n[3]
3% Starch	2.89 ± 0.63 [a]	18
3% Starch + 3% maltose	2.61 ± 0.74 [a]	3
3% Starch + 3% xylose	2.06 ± 0.28 [a]	3
3% Starch + 3% lactate	7.49 ± 2.10 [b]	14
3% Starch + 3% pyruvate	5.06 ± 0.60 [b]	3
3% Lactate	0.86 ± 0.34 [c]	15

1) FB_2 produced (average ± standard deviation) by *A. niger* IBT 28144 after 66-67 hours on media supplemented with the indicated carbon sources.
2) Different letters indicate statistically significant differences using Fisher's least significant difference procedure (95% confidence).
3) Number of replicates.

concentration of repressing sugar [31]. The available energy contributed from 3% lactate is expected to be a bit lower than from 3% starch, as less ATP is generated from 2 lactate (eq. 1 and 2) than from 1 glucose (eq. 3). But, this is based on the assumption that a full conversion of starch to glucose occurs and that glucose is not turned into energy storage metabolites as trehalose or polyols, as it does during liquid culture conditions [32].

$$\text{L-Lactate} + NAD^+ \rightarrow \text{Pyruvate} + NADH + H^+ \quad (1)$$

$$\text{L-Lactate} \rightarrow \text{Pyruvate} + 2e^- + 2H^+ \quad (2)$$

$$1/2\ \text{D-Glucose} + NAD^+ + P_i + ADP \rightarrow\rightarrow\rightarrow \text{Pyruvate} + NADH + ATP \quad (3)$$

In practice, we observed a low biomass production (mg dry weight/cm^2) on the medium with 3% lactate, while the produced biomass on media containing 3% starch with or without additional 3% lactate was not significantly different. Although the presence of starch was important for both growth and FB_2 production of *A. niger*, addition of either 3% maltose or 3% xylose to medium containing 3% starch did not further increase the FB_2 production. The effect of added lactate can consequently not be a simple result of a double amount of carbon source.

Exploring the proteome

Proteome analysis was conducted in order to identify proteins for which expression levels were altered during growth of *A. niger* on media containing 3% starch (S), 3% starch + 3% lactate (SL) and 3% lactate (L), and if possible relate the identified proteins to the influence on FB_2 production. The samples for protein extraction were taken 60 hours after inoculation as the FB_2 production rate was estimated to be highest at this time. In order to document FB_2 synthesis, FB_2 production was measured after 58 hours and 66 hours. The FB_2 synthesis rate was calculated to be (average ± 95% confidence limits, n = 6) 280 ± 140 ng/cm^2/h on S, 520 ± 90 ng/cm^2/h on SL and 10 ± 60 ng/cm^2/h on L. Biomass (dry weight) was measured after 62 hours and was (average ± standard deviations, n = 3) 6.2 ± 0.4 mg/cm^2 on S, 6.5 ± 1.0 mg/cm^2 on SL and 1.3 ± 0.3 mg/cm^2 on L.

Extracted proteins were separated by two-dimensional polyacrylamide gel electrophoresis (Figure 4). On 18 gels, representing 2 biological replicates and 3 technical replicates of *A. niger* cultures on each of the media S, SL and L, we detected 536-721 spots. With regard to the size of gels and amount of loaded protein, this was comparable to detected spots in other proteome studies of intracellular proteins in *Aspergillus* [33,34]. One protein was present at very high levels on the media containing starch, which was identified as glucoamylase [Swiss-Prot: P69328]. Jorgensen et al. [35] did similarly find this protein to have the highest transcript level of all genes in a transcriptome analysis of *A. niger* on maltose. Because of the volume and diffusion of this spot, the area containing this spot was excluded from the data analysis. About 80% of the spots were matched to spots on a reference gel containing a mixture of all samples. Thus, the total dataset for further analysis consisted of 649 matched spots (see Additional file 1).

Large differences in the proteome of *A. niger* when grown on S, SL and L were evident. A principal component analysis (PCA) clearly separated the gels with proteins from each media into three separate groups (Figure 5). The largest variance in relative spot volume was between samples from media with or without presence of starch (1^{st} component), while the next-largest variance in relative spot volume separated samples from S and SL (2^{nd} component). Statistically, 36% of the spots were present at significantly different levels between two or all three of the treatments (two-sided Students t-test, 95% confidence). Clustering of the 649 spots according to their relative spot volume by consensus clustering [36] resulted in prediction of 39 clusters. More than half of the spots were in clusters with a clear influence of medium on the protein level (18 clusters corresponding to 53% of the spots, Table 2) and 130 spots were in clusters with protein levels affected specifically on SL (cluster (cl.) 4, 7, 8, 35, 36, 37, 38).

The spots to be identified were selected within clusters with a profile with either distinct or tendency for higher (Table 3) or lower (Table 4) protein levels on SL compared to on S and L as these correlated positively or negatively with FB_2 production. Also some spots with levels influenced by presence of starch (Table 5) or lactate (Table 6) with either distinct or highly abundant presence on the gels were selected. Spots present at significant different levels between the two or three treatments were preferred. A total of 59 spots were identified using in-gel trypsin digestion to peptides, MALDI TOF/TOF and Mascot searches of retrieved MS/MS spectra to sequences from the databases Swiss-Prot [37] or NCBInr [38]. We did not use any taxonomic restrictions, however all except one protein were confidently identified as *A. niger* (predicted) proteins. One protein (6715) that did not match an *A. niger* protein, probably because it was missed or truncated during sequencing, had a significant match to a protein from *N. crassa* [UniProt: NCU04657]. Only 6 proteins (8 spots) were identified as proteins in the Swiss-Prot database and thus regarded as fully characterised. Otherwise, the proteins were registered in the NCBInr database as it contains the protein entries predicted from

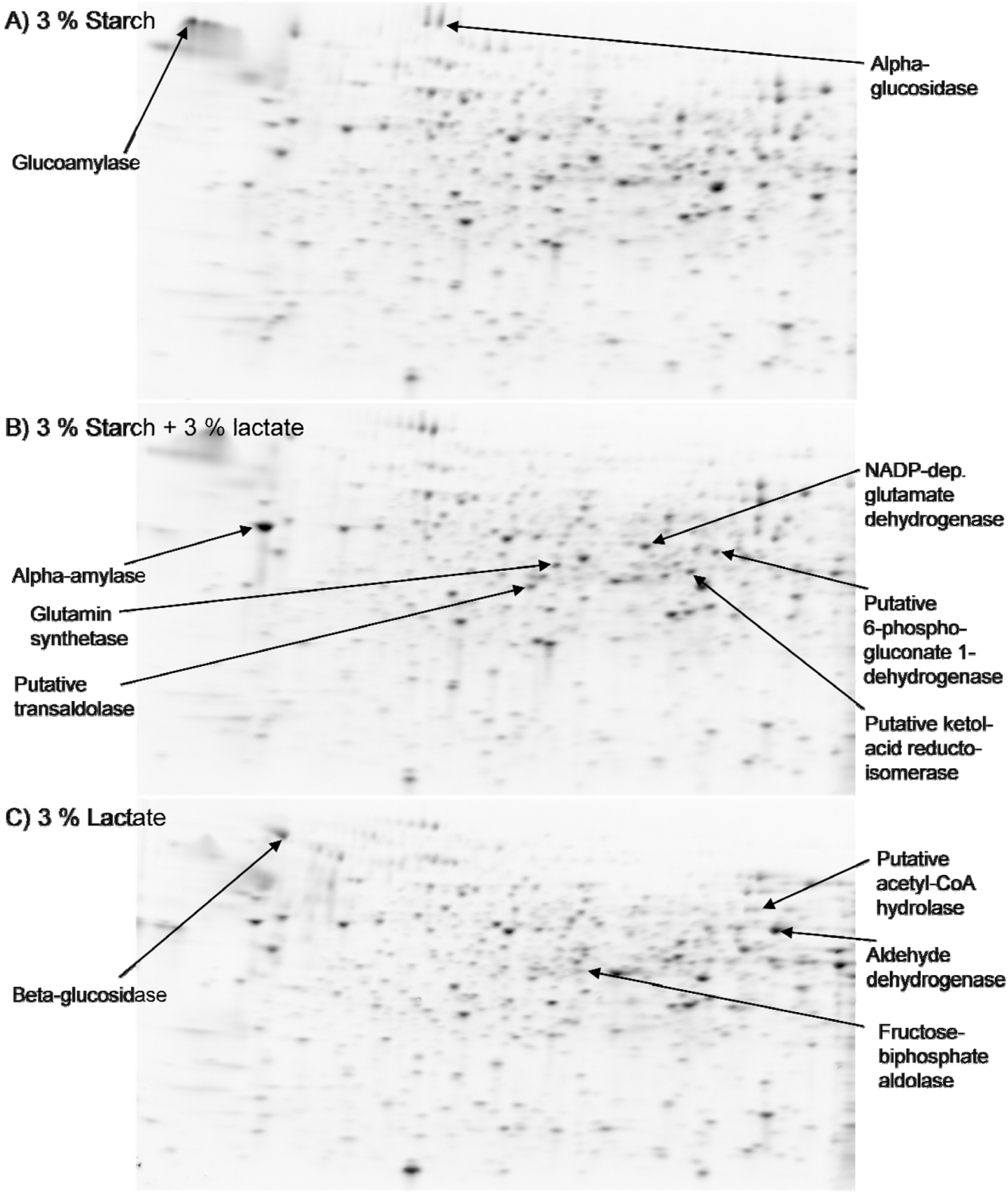


Figure 4
Example of representative 2D PAGE gels. 2D PAGE gels of proteins from *A. niger* IBT 28144 after 60 hours growth on media containing 3% starch (top), 3% starch + 3% lactate (middle) and 3% lactate (bottom).

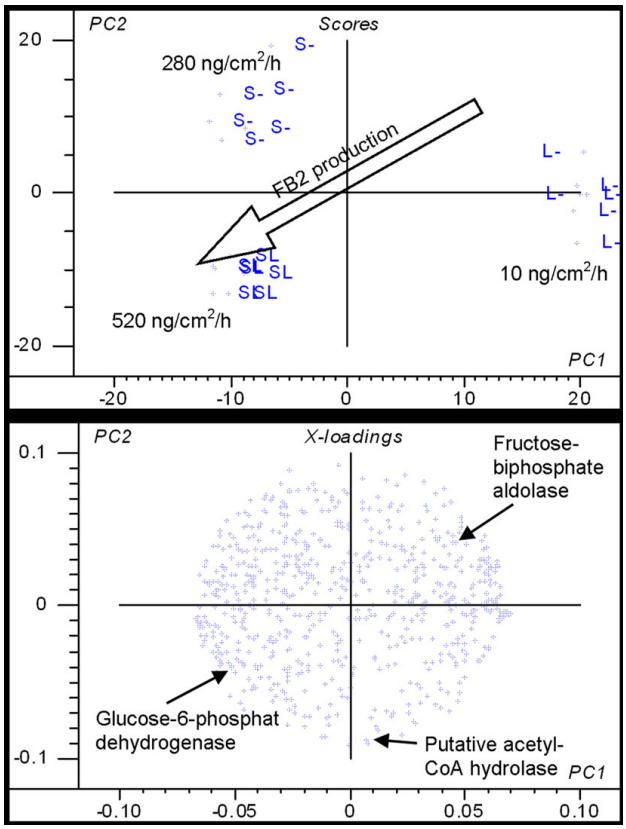


Figure 5
Illustration of variance in expressed proteins. Scoreplot (top) and loadingplot (bottom) from a principal component analysis of relative spot volume of all matched spots from the proteome analysis of *A. niger*. Shown is the 1st and 2nd principal component that explain 29% of the variance using validation with systematic exclusion of biological replicates.

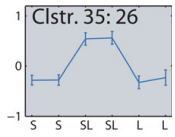
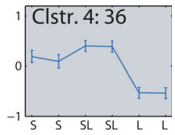
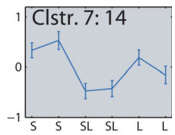
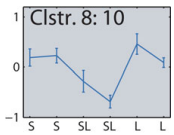
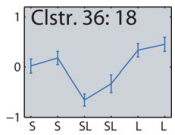
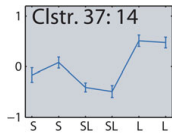
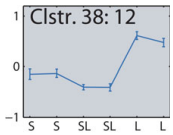
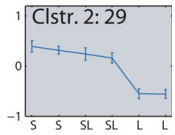
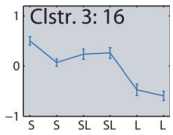
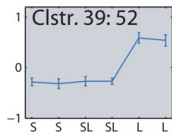
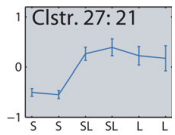
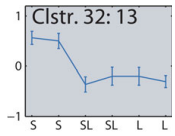
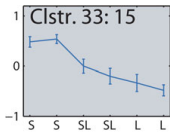
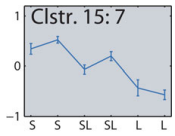
the sequencing of the *A. niger* CBS 513.88 genome [22]. Per primo March 2009 the predicted proteome based on this sequencing project contained 13906 predicted proteins of which 47.1% had automatically assigned GO annotations and only 154 proteins had been assigned as manually reviewed in the UniProtKB database [39]. To circumvent the limited number of annotated proteins, we assigned annotations based on sequence similarity to characterised Swiss-Prot proteins in other species using BlastP [40]. A protein annotation was assigned to a protein if it had more than 80% sequence identity to a characterised Swiss-Prot protein and a "putative" annotation to proteins that had 50-80% sequence identity to a characterised protein. Other proteins were assigned a "predicted" function if InterPro domains were predicted using InterProScan [41]. In this way, the identified proteins consisted of 6 (8 spots) fully characterised, 12 with annotation based on sequence similarity, 19 with putative annotation, 13 with predicted function and 6 (7 spots) uncharacterised proteins. The proteins with known functions were mainly involved in processes as: polysaccharide degradation; carbon-, nitrogen- and amino acid metabolism; energy production; protein synthesis, folding and degradation; redox balance and protection against oxidative stress. None of the characterised proteins were known to participate in secondary metabolite biosynthesis. A fatty acid synthase subunit alpha [UniProt: A2Q7B6] was identified, which was present at higher levels on SL compared to on S and L (cl. 35). This protein may contribute to fatty acid biosynthesis to be incorporated in the cell membrane; however it may also be an unrecognised polyketide synthase. One gene coding for a predicted aldo/keto reductase [UniProt: A2Q981] was located adjacent to the predicted FB_2 biosynthesis cluster in the *A. niger* genome. But this protein was present at higher levels on starch-containing media (cl. 3) and therefore did not correlate with FB_2 production. Furthermore, proteins involved in secondary metabolite synthesis or processes associated with transport or self-protection are not necessarily located within the clusters. One example is a reductase found to participate in aflatoxin biosynthesis in *A. parasiticus*, although it is not located within the aflatoxin cluster and was regulated differently than the aflatoxin cluster genes [42].

A throughout tendency was that many of the proteins influenced by the combination of starch and lactate in the medium were likely to affect either the acetyl-CoA level or the NADPH level as discussed below.

Regulation of central metabolic enzymes

The identified proteins appeared to include several important enzymes in the primary metabolism (Figure 6). Glucose 6-phosphate 1-dehydrogenase [Swiss-Prot: P48826] and a putative 6-phosphogluconate dehydrogenase [UniProt: Q874Q3], the first (rate-controlling) and third enzyme in the oxidative part of the pentose phosphate pathway (PPP) were present at higher levels on SL (cl. 35). They both reduce NADP to NADPH, and these enzymes are believed to be the main source of NADPH regeneration in the cell [43-46]. Additionally three enzymes in the non-oxidative part of the PPP were identified. A putative transketolase [UniProt: Q874Q5] and a putative transaldolase [UniProt: A2QMZ4] had tendencies for higher levels on SL (cl. 4). A predicted ribose/galactose isomerase [UniProt: A2QCB3], presumably with ribose 5-phosphate isomerase activity, was present at lower levels on SL (cl. 36). Lower level of this enzyme, responsible for synthesis of ribose 5-phosphate required for the biosynthesis of some amino acids, nucleotides, and coenzymes, indicates that the PPP was optimised to NADPH regeneration rather than to

Table 2: Clusters and interpretation

Description of clusters	Cluster profiles[1]			No. of spots Total	Identified
Higher levels on SL				26	11
Tendency for higher levels on SL				36	16
Lower levels on SL				42	4
Tendency for lower levels on SL				26	16
Higher levels if starch is present				45	3
Lower levels if starch is present				52	0
Higher levels if lactate is present				21	4
Lower levels if lactate is present				35	0
Possibly an effect, instability	Clusters 11, 16, 26, 30			58	3
No effect, instability and noise	Clusters 1, 5, 6, 9, 10, 12, 13, 14, 17, 18, 19, 20, 21, 22, 23, 24, 25, 28, 29, 31, 34			308	1
Total				649	58[2]

1) The graphs show the protein level profiles for selected clusters shown as transformed values between -1 and 1, where 0 indicates the average protein level. The bars give the standard deviations within the clusters.
2) One spot, identified as glucoamylase [Swiss-Prot: P69328], was excluded from the data analysis (see text). Thus the total number of identified spots was 59.

nucleotide synthesis on SL. One glycolysis enzyme, fructose-biphosphate aldolase [UniProt: A2QDL0], had tendency for lower level on SL (cl. 37), which is in good agreement with a higher activity of the PPP. Those enzymes identified downstream of pyruvate, the entry point of lactate into metabolism, were either clearly present at higher levels on SL or had the tendency for higher level. This included a putative pyruvate

Table 3: Identified proteins with higher levels on medium with starch + lactate

Protein	Spot		Identification[1]							Expression	
Annotation[2]	**Id.**	**Mass kDa[3]**	**Database**	**Acc. no.**	**Mass kDa**	**pI**	**MP**	**Score**	**SC %**	**CI. no.**	**Profile**
Alpha-amylase, extracellular	6601	53	NCBInr	A2QL05	55[6]	4.5	5	315	13	35	
Fatty acid synthase subunit alpha	6465	76[4]	NCBInr	A2Q7B6	205	5.9	10	387	5	35	
Glucose-6-phosphate 1-dehydrogenase	6561	59	Swiss-Prot	P48826	59	6.2	3	130	7	35	
Glutamine synthetase	6714	42	NCBInr	A2Q9R3	42	5.5	4	290	16	4	
Heat shock protein Hsp70	6481	73	NCBInr	A2QPM8	70	5.1	5	198	12	4	
Isocitrate dehydrogenase [NADP], mitochondrial, precursor	6644	48	Swiss-Prot	P79089	56	8.5	8	339	14	19	
NADP-dependent glutamate dehydrogenase	6647	48	NCBInr	A2QHT6	50	5.8	6	382	18	4	
Predicted 2-nitropropane dioxygenase	6737	41	NCBInr	A2QKX9	38[6]	5.7	4	112	17	35	
Predicted glucose-methanol-choline (Gmc) oxidoreductase	6515	65	NCBInr	A2R501	65	5.4	6	373	18	35	
Predicted methyltransferase	6810	36	NCBInr	A2QNF3	37	5.9	5	200	21	30	
Predicted NADH cytochrome b5 reductase	6693	44	NCBInr	A2R2Z2	46	5.4	6	530	20	4	
Predicted ubiquitin conjugating enzyme	7044	17	NCBInr	A2QDZ9	17	5.5	2	105	18	4	
Putative 6-phosphogluconate dehydrogenase, decarboxylating	6660	47	NCBInr	Q874Q3	55	5.9	9	527	27	35	
Putative aconitate hydratase, mitochondrial	6472	75	NCBInr	A2QSF4	84	6.2	7	278	11	35	
Putative heat shock protein Ssc1, mitochondrial	6487	71	NCBInr	A2R7X5	72	5.6	5	282	9	4	
Putative histidine biosynthesis trifunctional protein	6413	101[5]	NCBInr	A2QAS4	92	5.4	2	147	3	4	
Putative inositol-1-phosphate synthase	6573	57	NCBInr	A2QV05	58	5.7	2	62	4	35	
Putative ketol-acid reductoisomerase, mitochondrial	6730	41	NCBInr	A2QU08	45[6]	8.9	8	467	17	35	
Putative oxoglutarate dehydrogenase	6408	101[5]	NCBInr	A2QIU5	119	6.3	10	349	8	35	
Putative peroxiredoxin pmp20, peroxisomal membrane	7000	22	NCBInr	A2R0G9	19	5.4	8	610	54	4	
Putative peroxiredoxin Prx1, mitochondrial	6944	28	NCBInr	A2QIF8	23	5.2	5	224	22	4	
Putative pyruvate dehydrogenase E1 component subunit alpha, mitochondrial precurser	7028	18[4]	NCBInr	A2QPI1	45	7.6	2	160	7	30	
Putative transaldolase	6787	38	NCBInr	A2QMZ4	36	5.6	5	319	20	4	

Table 3: Identified proteins with higher levels on medium with starch + lactate *(Continued)*

Putative transketolase	6471	75	NCBInr	Q874Q5	75	6.0	6	246	11	4	
Thioredoxin reductase	6680	45	NCBInr	A2Q9P0	39	5.2	6	449	22	4	
Uncharacterised protein	6965	26	NCBInr	A2QDU1	19	5.4	3	147	15	4	
Uncharacterised protein	6591	55	NCBInr	A2QDX8	57	5.8	10	601	23	4	
Uncharacterised protein	6592	55	NCBInr	A2QDX8	57	5.8	10	717	25	4	
Uncharacterised protein	7059	16	NCBInr	A5ABN7	26	10.3	2	145	14	35	
Uncharacterised protein	7092	13[5]	NCBInr	A2QSA8	13	5.2	2	249	35	4	

List of identified proteins showing from left to right: Protein name, spot id and observed mass on gels, database, UniProt KB accession number, expected mass and isoelectric point (pI), number of matching peptide sequences (MP), Mowse Score (Score) and sequence coverage (SC), cluster and graph showing protein levels (average relative spot volume ± standard deviation) on media containing 3% starch (left/blue), 3% starch + 3% lactate (middle/purple) and 3% lactate (right/red).

1) Identification was based on Mascot MS/MS Ion Search using sequence data from the databases Swiss-Prot or NCBInr. Protein matches with significant ($p < 0.05$) Mowse Scores and ≥ 2 matching peptides were regarded as possible candidates for identification.

2) Annotation of uncharacterised proteins was based on sequence homology to characterised Swiss-Prot proteins using BlastP. Proteins were given a full annotation if they had > 80% sequence identity to a characterised Swiss-Prot protein or a putative annotation if they had 50-80% sequence identity to a characterised protein. Remaining proteins were assigned a "predicted" function if InterPro domains were predicted using InterProScan.

3) Observed mass on reference gel calibrated with molecular weight standards (14.4-97.4 kDa).

4) The spot is most likely a fragment as the retrieved peptides were localized in one of the ends of the protein sequence.

5) Mass above or below calibration range.

6) The protein is predicted to contain a signal peptide.

7) The protein is predicted to be glycosylated.

dehydrogenase (E1 subunit alpha) [UniProt: A2QPI1] (cl. 30) and the three enzymes in the tricarboxylic acid (TCA) cycle converting citrate to isocitrate, the irreversible step from isocitrate to 2-oxoglutarate, and from 2-oxoglutarate to succinyl-CoA. The first and the third TCA cycle enzyme, a putative aconitate hydratase [UniProt: A2QSF4] and a putative 2-oxoglutarate dehydrogenase [UniProt: A2QIU5], was clearly present at higher levels on SL (cl. 35), while NADP-dependant isocitrate dehydrogenase [Swiss-Prot: P79089] had a tendency for higher level but with a noisy profile (cl. 19). One enzyme that occurred at higher level when lactate was present in the media (cl. 27) was a putative acetyl-CoA hydrolase [UniProt: A2R8G9]. This enzyme has been designated to catalyse the hydrolysis of acetyl-CoA to acetate, but may rather posses CoA transferase activity between succinyl-, propionyl- and acetyl-CoA and the corresponding acids [47]. In yeast, acetyl-CoA hydrolase is involved in trafficking of acetyl-CoA across membranes in the form of acetate and thus is expected to be important for regulation of the acetyl-CoA level [48,49].

To summarize, higher levels of the enzymes in the PPP that generate NADPH during growth on SL compared to on S and L indicate an increased ability to regenerate NADPH when the NADP:NADPH ratio is increased. The higher levels of the enzymes in the metabolism of pyruvate after pyruvate enters mitochondria on SL and the higher levels of putative acetyl-CoA hydrolase in presence of lactate indicate an increased amount of carbon passing through acetyl-CoA during growth on SL.

Regulation of enzymes influencing the NADPH level

A remarkable requirement for NADPH on SL medium is pointed out by the simultaneous effect on several of the relatively few enzymes that contribute to NADPH regeneration. We found glucose 6-phosphate dehydrogenase, putative 6-phosphogluconate dehydrogenase, NADP-dependent isocitrate dehydrogenase and putative ketol-acid reductoisomerase [UniProt: A2QUO8], an enzyme in isoleucine, leucine and valine biosynthesis, to be present at higher levels on SL. Regulation of these enzymes is probably due to an increased NADP:NADPH ratio. The activity of the first enzyme, glucose 6-phosphate dehydrogenase, is known to be regulated by NADP:NADPH levels [50]. Larochelle et al. [51] showed in yeast that transcription of the corresponding gene was also affected by the NADPH level and they attributed this to a transcription factor Stb5. The yeast cell regulates the metabolism to counteract a high NADP:NADPH ratio by up-regulating the PPP and down-regulating glycolysis [51], which neatly corresponds to the changes we have observed in these pathways.

A. niger needs a supply of NADPH for several anabolic and biosynthetic processes as well as for protection

Table 4: Identified proteins with lower levels on medium with starch + lactate

Protein	Spot		Identification[1]							Expression	
Annotation[2]	**Id.**	**Mass kDa[3]**	**Database**	**Acc. no.**	**Mass kDa**	**pI**	**MP**	**Score**	**SC %**	**Cl. no.**	**Profile**
Aldehyde dehydrogenase	6605	53	Swis-Prot	P41751	54	6.0	10	908	34	37	
Aldehyde dehydrogenase	6615	52	Swis-Prot	P41751	54	6.0	7	646	20	38	
Beta-glucosidase 1 precurser	6360	130[5]	NCBInr	Q30BH9	94	4.7	5	267	6	36	
Fructose-biphosphate aldolase	6766	39	NCBInr	A2QDL0	40	5.5	8	697	28	37	
Predicted estherase/lipase/ thioesterase	6451	82	NCBInr	A2QTP5	84	5.4	9	543	18	37	
Predicted fumaryl-acetoacetate hydrolase	6663	47	NCBInr	A2QIN6	45	5.2	6	611	24	38	
Predicted glutathione-S-transferase	6952	27	NCBInr	A2R874	24	5.1	5	391	31	37	
Predicted NAD-dependant epimerase/dehydratase	6707	43	NCBInr	A2R992	38	5.7	7	397	26	38	
Predicted ribose/galactose isomerase	7035	18	NCBInr	A2QCB3	17	7.7	7	593	61	36	
Predicted Zn-containing alcohol dehydrogenase	6718	42	NCBInr	A2QAN5	39	5.8	4	298	19	38	
Putative 1-aminocyclopropane-1-carboxylate deaminase	6715	42	NCBInr Cross sp.	Q7S3B7	39	5.8	2	115	11	38	
Putative glutamate carboxypeptidase-like	6609	53	NCBInr	A2QY36	53	5.2	12	811	29	38	
Putative HIT family protein 1	7091	13[5]	NCBInr	A2QLN7	15	6.3	3	227	40	37	
Putative H-transporting two sec tor ATPase subunit F, vacuolar	7083	14	NCBInr	A2QCE6	14	5.3	4	340	44	37	
Putative NADH ubiquinone reductase, 40 kDa subunit, mitochondrial	6738	41	NCBInr	A2QSH0	43	6.7	5	307	17	38	
Putative peroxiredoxin pmp20, peroxisomal membrane	7031	18	NCBInr	A2R6R3	18	5.6	5	431	37	38	
Superoxide dismutase Cu-Zn, cytoplasmic	7046	17	Swiss-Prot	A2QMY6	16	5.9	5	323	38	36	
Ubiquitin-like protein	7113	11[5]	NCBInr	A2QKN1	9	5.8	5	272	60	37	
Uncharacterised protein	7002	21	NCBInr	A2QLX7	20	6.1	7	592	55	8	
Uncharacterised protein	7074	15[4]	NCBInr	A2QBG0	34	5.1	6	609	24	38	

See legend and notes to table 3.

Table 5: Identified proteins with levels influenced by presence of starch

Protein	Spot		Identification[1]							Expression	
Annotation[2]	**Id.**	**Mass kDa[3]**	**Database**	**Acc. no.**	**Mass kDa**	**pI**	**MP**	**Score**	**SC %**	**Cl. no.**	**Profile**
Alpha-glucosidase, extracellular	6354	151[5]	Swiss-Prot	P56526	109	5.1	7	497	10	2	
Glucoamylase isoform G1, glycosylated	6000	130[5]	Swiss-Prot	P69328	69[6,7]	4.3	5	308	10	-	-
Predicted aldo/keto reductase	6781	38	NCBInr	A2Q981	37	6.0	5	335	17	3	
Pyruvate decarboxylase	6540	61	NCBInr	A5AA75	63	6.3	6	412	15	3	
Translation elongation factor 2	6836	35[4]	NCBInr	A2QD36	94	6.5	6	556	7	11	

See legend and notes to table 3.

Table 6: Identified proteins with levels influenced by presence of lactate

Protein	Spot		Identification[1]							Expression	
Annotation[2]	Id.	Mass kDa[3]	Database	Acc. no.	Mass kDa	pI	MP	Score	SC %	Cl. no.	Profile
Alpha-glucfosidase, extracellular	6355	157[5]	Swiss-Prot	P56526	109	5.1	3	147	4	27	
Predicted NMR-like protein	6783	38	NCBInr	A2R745	34[6]	5.2	3	225	14	27	
Putative acetyl-CoA hydrolase, glycosylated	6533	62	NCBInr	A2R8G9	58[7]	6.0	5	253	10	27	
Putative NADH ubiquinone reductase, 31 kD subunit	6888	32	NCBInr	A2QWS1	32	7.7	2	104	8	27	

See legend and notes to table 3.

against oxidative stress. A supply of NADPH is for example required in order to utilize nitrate as nitrogen source, since the enzyme that converts nitrate to nitrite, nitrate reductase, uses NADPH as cofactor [44].

On SL, we observed higher levels of enzymes involved in fatty acid biosynthesis, ammonium assimilation and protection against oxidative stress, those activities may increase the NADP:NADPH ratio [52]. As mentioned previously, we observed a higher level of a fatty acid synthase subunit alpha on SL (cl. 35) that requires NADPH in order to catalyse the biosynthesis of fatty acids. We also identified NADP-dependant glutamate dehydrogenase [UniProt: A2QHT6] involved in ammonium assimilation and thioredoxin reductase [UniProt: A2Q9P0] that utilises NADPH to reduce thioredoxin during conditions with oxidative stress; both had tendencies for higher levels on SL (cl. 4). Furthermore, the polyketide synthase involved in FB_2 biosynthesis uses NADPH as cofactor [13] and that may also affect the NADP:NADPH ratio.

These results show a clear tendency towards increased NADPH turnover and regeneration during growth on SL.

Relation between regulated proteins and FB_2 biosynthesis

The identified proteins regulated on SL were mainly enzymes in the primary metabolism and other processes that likely affect the intracellular levels of acetyl-CoA or NADPH. The higher FB_2 production on SL is thus most likely a result of changes in the metabolism due to lactate degradation. Acetyl-CoA is a precursor for production of FB_2 as well as for other polyketide-derived metabolites [13]. High level of acetyl-CoA during growth on SL may thus be what drives the high FB_2 production. This is supported by the observation that pyruvate had a similar effect as lactate on FB_2 production. A good ability to regenerate NADPH when the NADP:NADPH ratio is increased may be an important prerequisite for the high FB_2 production on SL.

However, the effect of added lactate to a medium containing starch on FB_2 production was dramatic and not expected to be solely precursor-driven. Further, the 12 secondary metabolites measured in this study, which include polyketides, non-ribosomal peptides and polyketide-derived alkaloids, were affected differently by the presence of starch and lactate and a pattern reflecting the biosynthetic origin of the metabolites was not evident. This supports that the influence of lactate in combination with starch on FB_2 production is regulatory rather than an effect solely driven by abundance of precursors. We hypothesise that the FB_2 production, when induced, could be regulated globally according to the nutrient/energy state. As a central compound in metabolism, carefully regulated and compartmentalised, acetyl-CoA may be a candidate for this [53]. Acetyl-CoA has been shown to be able to affect transcription in vitro [54]. In yeast, it has been suggested that transcription of the inositol 1-phosphate synthase gene, *ino1*, is influenced by the acetyl-CoA level during conditions with high levels of energy-rich metabolites [55]. In accordance, we identified a putative inositol-1-phosphate synthase [UniProt: A2QV05] among the proteins with higher levels on SL medium (cl. 35). Inositol-1-phosphate synthase is the first and rate-controlling enzyme in the inositol biosynthesis pathway and converts glucose 6-phosphate into inositol 1-phosphate. Inositol is incorporated into phosphatidylinositol that in turn is a precursor of sphingolipids and inositol polyphosphates, required for a diverse set of processes that include glycolipid anchoring of proteins, signal transduction (regulation of chromatin remodeling and transcription), mRNAexport and vesicle trafficking [56,57]. Acetyl-CoA is also a substrate for protein acetylation by protein acetylases, and acetylation can influence both gene expression and protein activity [58]. In *A. parasiticus* there has been observed a correlation between initiation and spread of histone acetylation in the aflatoxin gene promoters and the initiation of aflatoxin gene expression [59]. Another study of *A. nidulans* has shown that genetic deletion of a histone deacetylase caused elevated gene

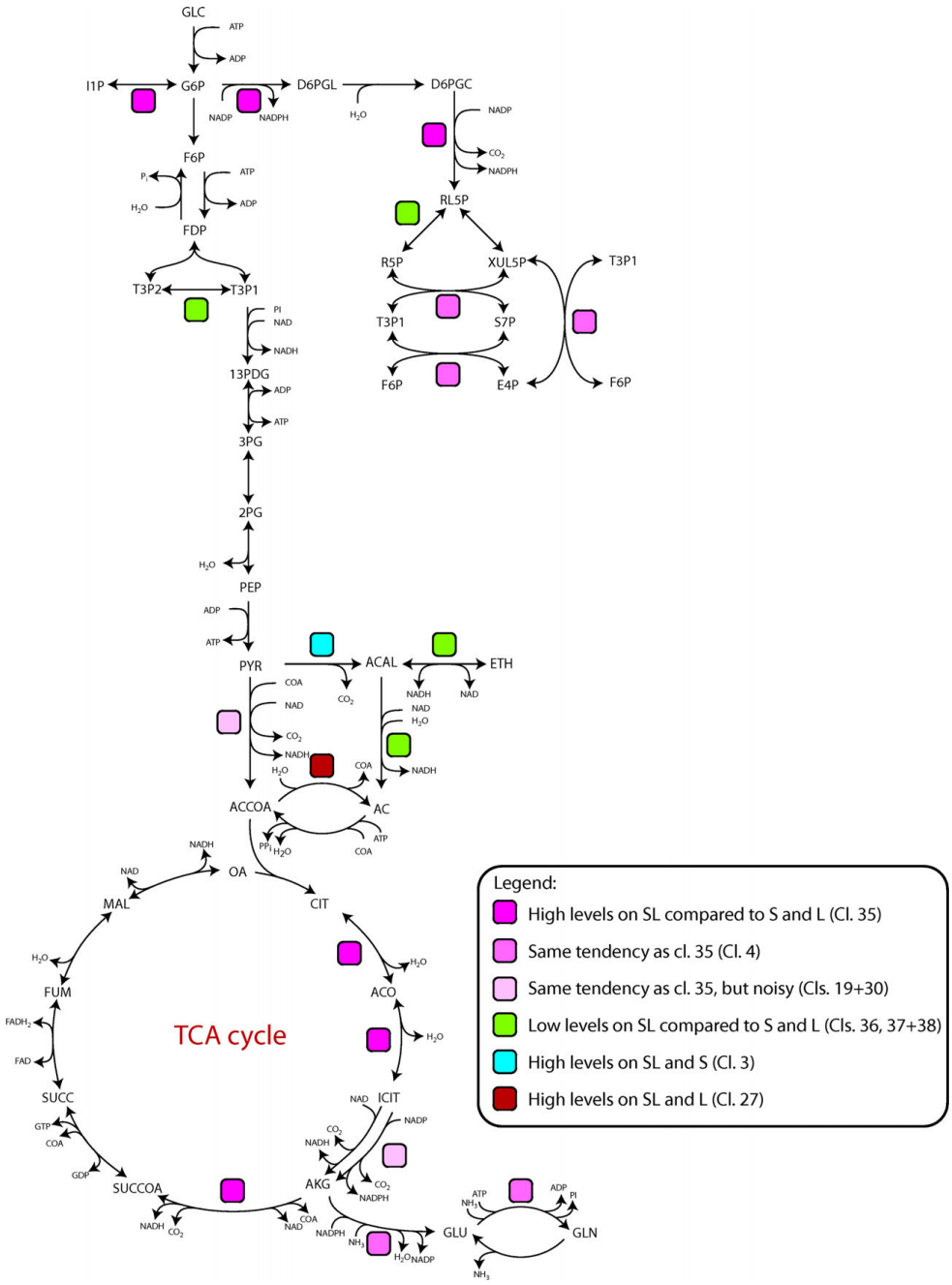


Figure 6

Identified proteins within the primary metabolism. Pathway map showing an outline of the glycolysis, the pentose phosphate pathway, pyruvate metabolism, the tricarboxylic acid cycle and ammonium assimilation enzymes with the identified proteins indicated. Modified from map of *A. niger* metabolism published by Andersen et al [68]. 13PDG: 1,3-bisphospho-D-glycerate, 2PG: 2-phospho-D-glycerate, 3PG: 3-phospho-D-glycerate, AC: acetate, ACAL: acetaldehyde, ACCOA: acetyl coenzyme A, ACO: cis-aconitate, AKG: 2-oxoglutarate, CIT: citrate, D6PGC: 6-phospho-D-gluconate, D6PGL: d-glucono-1,5-lactone 6-phosphate, E4P: D-erythrose 4-phosphate, ETH: ethanol, F6P: beta-D-fructose 6-phosphate, FDP: beta-D-fructose 1,6-bisphosphate, FUM: fumarate, G6P: alpha-D-glucose 6-phosphate, GLC: alpha-D-glucose, GLN:L-glutamine, GLU: L-glutamate, I1P:1D-inositol 3-phosphate, ICIT: isocitrate, MAL: (S)-malate, OA: oxaloacetate, PEP: phosphoenolpyruvate, PYR: pyruvate, R5P: D-ribose 5-phosphate, RL5P: D-ribulose 5-phosphate, S7P: sedoheptulose 7-phosphate, SUCC: succinate, SUCCoA: succinyl coenzyme A, T3P1: D-glyceraldehyde 3-phosphate, T3P2: glycerone phosphate (DHAP), XUL5P:D-xylulose 5-phosphate.

expression and enhanced production of sterigmatocystin and penicillin [60]. The same study demonstrated that treatment with histone deacetylase inhibitors could enhance production of some secondary metabolites by *Penicillium expansum* and *Alternaria alternata*, indicating that histone acetylation and deacetylation have a role in regulation of secondary metabolite production in a broad range of fungal genera.

Secondary metabolite synthesis can be subject to multiple regulatory mechanisms. Regulation of fumonisin B_1 biosynthesis in *F. verticillioides* has been found to be complex with several positive and negative regulators and influenced by nitrogen, carbon and pH [12,61]. Corresponding to our results, fumonisin B_1 production in *F. verticillioides* has been shown to be induced by the presence of starch [62]. However, *F. verticillioides* and *A. niger* are widely different physiologically and genetically, thus production and regulation of fumonisin biosynthesis are not expected to be identical [6].

During conditions where *A. niger* spends resources on producing extracellular enzymes for degradation of plant tissue and starch, protection against other microorganisms competing for nutrients would be beneficial. Fumonisin B_1 has been shown to have antifungal activity against species as *Alternaria alternata*, *Penicillium expansum*, *Botrytis cinerea* and *Fusarium graminearum* [63], thus FB_2 could be expected to have a similar effect. Increased production of FB_2 during conditions with high acetyl-CoA level may thus have evolved because antifungal activity was advantageous to *A. niger* as a way to protect the nutrient sources in the environment.

Conclusions

Our results show that lactate, when supplemented in a rich substrate containing nitrate and starch, can increase the FB_2 production in *A. niger*. Based on the identified proteins within the central metabolism, we suggest this to be due to changes in the balance of intracellular metabolites towards a higher level of carbon passing through acetyl-CoA and a high capacity to regenerate NADPH. Given that the FB_2 biosynthesis genes are induced, the results indicate that the availability of precursors and NADPH has a large influence on production of FB_2. The production of certain other secondary metabolites was affected in a similar fashion as FB_2 by lactate (fumonisin B_4, orlandin, desmethylkotanin and pyranonigrin A), while other secondary metabolites were not (ochratoxin A, ochratoxin alpha, malformin A, malformin C, kotanin, aurasperone B, tensidol B). Consequently, as these metabolites were affected differently by the presence of starch and lactate, they must be regulated differently in *A. niger*.

We find it likely that the influence of starch and lactate/pyruvate on FB_2 production is part of a global regulation inferred by the nutrient/energy state and propose that this could be through the action of acetyl-CoA. Whether, if and how, acetyl-CoA affects gene transcription or activity of enzymes in the FB_2 biosynthesis pathway could be the scope of relevant, future studies.

It remains to be seen whether production of secondary metabolites in other species of filamentous fungi is increased by presence of starch and lactate. The effect of starch and lactate in combination may be relevant to be aware of for starch-containing foods and feeds where fungi occur concurrently with lactic acid fermentation, which could be the case in low-fat mould-fermented sausages, in fermented vegetable products and in silage. Technologically, the obtained knowledge of substrate influence on production of specific secondary metabolites could be beneficial, as lactate or other carbon sources could be used to increase metabolite production during industrial fermentation.

Methods

Strain

A. niger IBT 28144 (CBS 101705) was obtained from the IBT culture collection and maintained on silica gel. The culture was used after two successive inoculations on Czapek Yeast Autolysate agar (CYA), incubated 7 days in dark at 25°C.

Media

Media were modified from CYA and contained per L: 5 g Yeast extract (Biokar Diagnostics, Beauvais, France); 3 g $NaNO_3$; 1 g K_2HPO_4; 0,5 g KCl; 0,5 g $MgSO_4 \cdot 7H_2O$; 0,01 g $FeSO_4 \cdot 7H_2O$; 0,01 g $ZnSO_4 \cdot 7H_2O$; 0,005 g $CuSO_4 \cdot 5H_2O$ and 20 g agar (Sobigel, VWR - Bie & Berntsen A/S, Herlev, Denmark). Soluble potato starch, 60% potassium L-lactate solution, maltose monohydrate, D-xylose and/or sodium pyruvate (all Sigma Aldrich, St. Louis, Missouri, USA) were added according to the indicated percentages in w/v. Lactate, maltose, xylose and pyruvate and the remaining ingredients were sterilised separately, at 121°C for 15 min., cooled to 60°Cbefore the ingredients were mixed, adjusted to pH 5.5 with sterile filtered 2 M KOH or 5 M HCl and poured into petri dishes.

Inoculation and incubation

Conidium suspensions were prepared in spore suspension media (0.50 g Tween 80, 0.50 g agar to 1 L water), filtrated through Miracloth (Merck KGaA, Darmstadt, Germany) to remove mycelium fragments and adjusted to 10^6 conidia/ml. Each agar plate was surface inoculated

with 10^5 conidia using a drigalsky spatula. Incubation was in dark at 25°C.

Determination of growth

Biomass production was determined in triplicate for surface inoculated cultures on agar plates covered with a 0.45 μm polycarbonate membrane (Isopore™, Millipore, Billerica, Massachusetts, USA). The whole mycelium was collected and the dry weight was determined after drying at 100°C for 20-24 h.

Determination of conidium production

Eight agar plugs (4 mm in diameter) were dispensed in 4 ml peptone water (1 g peptone (Difco, BD, Franklin Lakes, New Jersey, USA) to 1 l destilled water) and replicate measures of the conidium concentration were determined in a Thoma counting chamber for triplicate cultures.

Extraction of secondary metabolites

The method described by Smedsgaard [29] with some modifications was used for secondary metabolite extraction. A sample of 8 agar plugs (4 mm in diameter) taken randomly from the plate was extracted with 1 ml methanol/dichloromethane/ethyl acetate (v/v/v 1:2:3) containing 1% (v/v) formic acid for 60 min using ultrasonication. The extract was transferred to a new vial and the solvent evaporated. The agar plug sample was re-extracted with 0.8 ml 75% methanol in water for 60 min using ultrasonication and the extract combined with the dry extract of first extraction. The residues were re-dissolved by whirley mixing followed by 10 min ultrasonication and the extracts were filtrated through 0.45 μm PTFE filters.

LC-MS and HPLC-FLD for determination of secondary metabolites

LC-MS was performed on an Agilent 1100 LC system (Agilent Technologies, Santa Clara, California, USA) with a 40°C, 50 mm × 2 mm i. d., 3 μm, Luna C18 II column (Phenomenex, Torrance, California, USA). The LC system was coupled to a single quadropole mass detector (LC/MSD VL, Agilent technologies) with an atmospheric pressure ionisation source and to a 200-700 nm diode array detector. A sample volume of 3 μl was injected and eluted at a flow rate of 0.3 ml/min using a water-acetonitrile gradient system starting from 15% acetonitrile that was increased linearly to 100% in 20 min and with a holding time of 2 min. Water and acetonitrile were buffered with 20 mM formic acid and 5 mM ammonium formiate (only water). The ion source was operated in positive mode with a capillary voltage at 3000 V and detection was done in full scan from m/z 100-1000, a peak width of 0.1 min and a cycle time of 1.06 sec. HPLC-FLD was performed on a similar LC system coupled to a fluorescence detector. Water and acetonitrile were buffered with 50 mM trifluoroacetic acid (TFA). Excitation and emission wavelengths were 333 nm and 460 nm respectively. Chemstation (Agilent) was used for data collection and evaluation. Detection was based on the extracted ion chromatogram of the ions $[M+H]^+$ or $[M+NH_3]^+$ or fluorescence emission chromatograms (Table 7). Standards were used for confirmation of identity if available. Otherwise the identity was confirmed by presence of characteristic ions or adducts in the MS spectrum and characteristic UV absorbance spectrum. Quantification of FB_2 was based on a calibration curve created from dilutions of a fumonisin B_2 standard (50.1 μg/ml, Biopure, Tulln, Austria) at levels from 0.5 to 25 μg/ml. The remaining metabolites were semi-quantified based on peak areas, calculated in percentage of highest average peak area value of triplicates within the study.

Sampling for proteome analysis

Duplicate samples for proteome analysis were taken from surface inoculated cultures on agar plates covered with a 0.45 μm polycarbonate membrane (Isopore™, Millipore). The whole mycelium mass was collected and frozen in liquid nitrogen.

Protein extraction

The method described by Kniemeyer et al. [64] with few modifications was used for protein extraction. The mycelium was homogenised with mortar and pestle under liquid nitrogen and 100 mg of the homogenate was collected. The protein was precipitated with acetone added with 13.3% (w/v) trichloroacetic acid and 0.093% (v/v) 2-mercaptoethanol at -20°C for 24 hours followed by centrifugation at 20.000 × g in 15 min at 4°C. Pellet was washed twice in acetone with 0.07% (v/v) 2-mercaptoethanol and air-dried for 10 min. Pellet was suspended in 600 μl sample buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.8% (v/v) ampholytes (Bio-Lyte 3/10, Bio-Rad, Hercules, California, USA), 20 mM DTE and 20 mM Tris (Tris-HCl buffer pH 7.5). The solution was incubated for 1 hour at 20°C and ultrasonicated for 10 min. The sample was centrifuged at 17.000 × g for 30 min, and the supernatant was collected and stored at -80°C. Protein concentration was determined using a 2-D Quant kit (GE Healthcare, Uppsala, Sweden).

2D polyacrylamide gel electrophoresis

Isoelectric focusing was done using immobilised pH gradient strips (11 cm, pH 4-7, ReadyStrip™, Bio-Rad). A sample volume corresponding to either 40 μg (image analysis gels) or 100 μg (preparative gels) protein was

Table 7: Detection parameters for selected *A. niger* secondary metabolites

Metabolite		Detection Method[1]		Rt[2]	Confirmation Std.	MS ions and adducts[1]	UV peak absorption wavelengths[3]
Fumonisin B_2	[6]	MS	$[M+H]^+$ = m/z 706	9.6	×	$[M+Na]^+$ = m/z 728	End[4]
Fumonisin B_4	[24]	MS	$[M+H]^+$ = m/z 690	10.5	-	-	End[4]
Ochratoxin A	[5]	FLD	Excitation: 333 nm, emission: 460 nm	10.3	×	-	216 nm (100), 250 nm (sh), 332 nm (20) [69]
Ochratoxin alpha	[70]	FLD	Excitation: 333 nm, emission: 460 nm	7.1	×	-	216 nm (100), 235 nm (sh), 248 nm (sh), 336 nm (22) [69]
Malformin A_1	[71]	MS	$[M+NH3]^+$ = m/z 547	10.5	×	$[M+H]^+$ = m/z 530, $[M+Na]^+$ = m/z 552	End[4]
Malformin C	[72]	MS	$[M+NH3]^+$ = m/z 547	10.9	×	$[M+H]^+$ = m/z 530, $[M+Na]^+$ = m/z 552	End[4]
Orlandin	[73]	MS	$[M+H]^+$ = m/z 411	7.5	-	$[M+Na]^+$ = m/z 433	Similar to kotanin
Desmethyl-kotanin	[30]	MS	$[M+H]^+$ = m/z 425	9.3	-	$[M+Na]^+$ = m/z 447	Similar to kotanin
Kotanin	[30]	MS	$[M+H]^+$ = m/z 439	11.4	×	$[M+Na]^+$ = m/z 461	208 nm (100), 235 nm (sh), 296 nm (sh), 308 nm (47), 316 nm (sh) [69]
Aurasperone B	[74]	MS	$[M+H]^+$ = m/z 607	11.5	-	$[M+Na]^+$ = m/z 629	233 nm (68), 270 nm (sh), 280 nm (100), 318 nm (24), 331 nm (24), 404 nm (15)[75]
Pyranonigrin A	[76]	MS	$[M+H]^+$ = m/z 224	1.7	-	$[M+NH4]^+$ = m/z 241, $[M+Na]^+$ = m/z 246	210 nm (100), 250 nm (51), 314 nm (68) [77]
Tensidol B	[78]	MS	$[M+H]^+$ = m/z 344	9.1	-	$[M+Na]^+$ = m/z 366	206 nm (100), 242 nm (44) [78]

List of secondary metabolites included in this study with reference of their production in *A. niger*. Detection method and retention time, available standards used for confirmation (marked by x) and additional MS and UV spectral information used for confirmation.
1) Values obtained from Antibase 2007 (Wiley, Hoboken, New jersey, USA).
2) Retention time in respective LC systems (OTA and OT-alpha analysis on separate HPLC system).
3) Parenthesis values are absorption in percent of maximum absorption, sh denotes a shoulder.
4) End: End absorption (< 200 nm).

diluted to a total volume of 200 µl in a rehydration buffer consisting of 7 M urea; 2 M thiourea; 2% (w/v) CHAPS; 0.5% (v/v) ampholytes (Bio-Lyte 3/10, Bio-Rad); 1% (w/v) DTT and 0.002% (w/v) bromophenol blue. Rehydration was done at 250 V for 12 hours at 20°C. Focusing was done at an increasing voltage up to 8000 V within 2 1/2 hour and hold until 35 kVh was reached, with a maximal current of 50 µA/IPG strip. The voltage was hold at 500 V until the IPG strips were frozen at -20°C. The IPG strips were equilibrated in buffer containing 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer pH 8.8. First, the cysteines in the sample were reduced in equilibration buffer added with 1% (w/v) DTT for 15 min, and when alkylated in equilibration buffer added with 4% (w/v) iodoacetamide for 15 min. PAGE was done at 200 V in 10-20% gradient gels (Criterion Tris-HCl Gel, 10-250 kD, 13.3 × 8.7 cm, Bio-Rad) using an electrode buffer containing 25 mM Tris, 1.44% (w/v) glycine and 0.1% (w/v) SDS. Image analysis gels were fixed in 50% (v/v) ethanol, 7% (v/v) acetic acid two times for 30 min and stained over night in SYPRO Ruby Protein Gel Stain (Invitrogen, Life Technologies, Carlsbad, California, USA). The gels were washed in 10% (v/v) ethanol, 7% (v/v) acetic acid for 30 min. and two times in Milli-Q water (Millipore) for 5 min. The gels were visualized with a CCD camera (Camilla fluorescence detection system, Raytest, Straubenhardt, Germany) equipped with excitation and emission filters and with an exposure time of 100 ms. Images were saved as 16 bit tif-files. Preparative gels were fixed in 15% (w/v) ammoniumsulphate, 2% (v/v) phosphoric acid, 18% (v/v) ethanol in water and stained with Coomassie Brilliant blue (0.02% (w/v) Brilliant blue G in fixing buffer) overnight and washed two times in Milli-Q water. Gels were prepared in triplicate for each biological sample for image analysis gels and a reference gel containing an equal mixture of all samples was included. A molecular weight standard (14.4 - 97.4 kDa, BioRad) was applied to the reference gel before PAGE for mass calibration.

Image analysis

Images were imported, inverted and analyzed with Imagemaster 2D platinum v. 5 (GE Healthcare). Spot detection parameters were adjusted for optimal spot detection (smooth = 2; min. area = 30; saliency = 20) and the spots were quantified as the relative spot volume (percent spot volume) within each gel. The spots from each gel were paired with detected spots on a reference gel containing a mixture of all samples. Matching of gels was done automatically after selection of a landmark spot in each gel.

Statistical analysis

Statistical differences in relative spot volumes between the treatments were determined by two-sided Students

t-tests (H_0: $\mu_1 = \mu_2$, H_A: $\mu_1 \neq \mu_2$) using Imagemaster 2D platinum. The null hypothesis was rejected if $t_{df = 2} \leq 4.303$ (95% confidence).

Statistical analysis of FB_2 production was done using Statgraphics Plus v. 4.0 (StatPoint Inc., Herndon, Virginia, USA).

Principal component analysis

Principal component analysis was done using Unscrambler v. 8.0 (Camo Process AS, Oslo, Norway). The dataset consisted of 18 gels (samples) and 649 spots (variables) and corresponding relative spot volumes. All variables were centred and weighted by (standard deviation)$^{-1}$. Validation was based on systematic exclusion of samples corresponding to a biological replicate.

Cluster analysis

Cluster analysis was done using the Matlab clustering algorithm "ClusterLustre" described by Grotkjær et al [36]. The relative spot volumes were transformed to Pearson distances prior to clustering (results in values between -1 and 1, where 0 indicates the average expression level). Cluster solutions with K = 3-50 clusters were scanned with 20 repetitions. For each repetition the most likely number of clusters was determined by the Bayesian Information Criteria.

In-gel digestion of proteins

In-gel digestion was done according to Shevchenko et al. [65] with some minor modifications: The protein spots were excised from Coomassie stained gels loaded with 100 µg protein. A piece of gel without staining was used as a negative control. The gel pieces were cut into approx. 1 mm^3 pieces and washed twice for 15 min., first with water and second with water/acetonitrile 1:1 (v/v). The gel particles were then washed in acetonitrile to dehydrate the gel (they shrunk and became white). A volume of 10 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 to cover the gel pieces was added and the proteins were reduced for 45 min at 56°C. After cooling, the DTT solution was replaced by the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and the reduced proteins were alkylated for 30 min. in the dark. The gel pieces were then washed with water, water/acetonitrile 1:1 (v/v) and acetonitrile to dehydrate the gel. Ice-cold digestion buffer containing 12.5 ng/µl trypsin in 50 mM NH_4HCO_3 was added to the gel pieces in a volume just sufficient to rehydrate the gel (5-10 µl). After 45 min incubation on ice bath the unabsorbed digestion buffer was removed and replaced by 20 µl of 50 mM NH_4HCO_3 buffer to cover the gel pieces. The proteins were digested overnight at 37°C. The buffer solution with protein digest was recovered and kept at -20°C.

Micropurification of peptides and loading on MALDI target

The peptide solutions were purified on nano-scale reversed-phase columns prior to mass spectrometric analysis by the method described by Gobom et al [66]. The columns were prepared by loading a few µl slurry of a reversed phase chromatographic medium (Poros R2 10 µm, Applied Biosystems) dissolved in acetonitrile into a partially constricted GelLoader pipette tip. The column was packed by applying pressure with a syringe giving a column height of 4-10 mm and equilibrated with 1% TFA. The peptide digest was loaded onto the column and desalted by washing with 1% TFA. The peptides were eluted with matrix solution containing 5 µg/µl α-cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% TFA directly in one droplet onto the MALDI target (Opti-TOF® 384 Well MALDI Plate Inserts, Applied Biosystems, California, USA).

MALDI TOF/TOF tandem MS

MALDI peptide mass spectra and MS/MS spectra of selected peptides were obtained on a 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems). External mass calibration was done using a tryptic digest of beta-lactoglobolin (m/z 837.48 and 2313.26) and in some cases peaks from trypsin auto-digestion peptides (m/z 842.51 and 2211.12) were used for internal calibration of the peptide mass spectra. MS and MS/MS mass spectra were obtained at a laser intensity of 3000 and 3600 respectively. Peak lists were generated with an in house macro (in the Protein Research Group at Department of Biochemistry and Molecular Biology, University of Southern Denmark) using Data Explorer (Applied Biosystems) and converted to .mgf files containing the combined data from MS and MS/MS spectra for a sample.

Protein identification

Mascot MS/MS Ions Search (Matrix Science [67]) was used to search for matching protein sequences within the databases Swiss-Prot (Swiss Institute of Bioinformatics [37]) or NCBInr (National Center for Biotechnology Information [38]). The search parameters were: enzyme digestion with trypsin, no taxonomic restriction, carbamidomethyl (C) as fixed modification, oxidation (M) as variable modification, $[M+1]^+$ peptide charge state, monoisotopic mass values, unrestricted protein mass, ± 70 ppm peptide mass tolerance, ± 0.6 Da fragment mass tolerance, maximum 1 missed cleavage pr. peptide. Protein matches to *Aspergillus niger* proteins and with significant ($p < 0.05$) Mowse Scores were regarded as

possible candidates for identification. The candidate(s) were further inspected for number of matching peptides (=2), the mass accuracy of the matching peptides, the sequence coverage and distribution of matching peptides in the obtained sequences. The reported miscleavage sites were inspected for presence of amino acids that affect the action of trypsin (proline, glutamic acid and aspartic acid or additional lysine/arginine). Finally the molecular weight and isoelectric point of the obtained protein match were compared to those observed on the gels. From samples with low intensity, peptides from keratin and trypsin were erased if necessary.

Protein annotation

Annotation of uncharacterised proteins was based on sequence similarity to characterised Swiss-Prot proteins using BlastP [40]. Proteins were given a full annotation if they had more than 80% sequence identity to a characterised Swiss-Prot protein or a putative annotation to proteins if they had 50-80% sequence identity to a characterised protein. Other proteins were assigned a "predicted" function if InterPro domains were predicted using InterProScan (European Bioinformatics Institute [41]).

Authors' contributions

LMS participated in design of the study, carried out the experimental work, the statistical and multivariate analysis and prepared the manuscript. RL participated in design of the study, contributed to the proteome analysis and revised the manuscript. MRA carried out the cluster analysis, participated in protein annotation and interpretation and revised the manuscript. PVN and JCF participated in design of the study and revision of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Protein expression data. *Additional file 1.xlsx (an excel file) contains relative spot volumes for spots detected and matched to a reference gel in the 2D gel based proteome analysis of A. niger IBT 28144 on the three media containing 3% starch (S), 3% starch + 3% lactate (SL) and 3% lactate (L). B1-B6 denotes the biological replicate, R1-R2 the electrophoresis run and Gel 1-21 the gel number.*

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Discussion

The aim of the **first part** of the study was to establish the mycobiota of some typical Danish meat products and determine if toxinogenic species were present. Two processing plants for fermented sausages and two for liver pâté were examined (manuscript 1). This study was highly relevant as no other studies of filamentous fungi associated to North European meat products had been published at that time. Since when a Norwegian study of smoked and non-smoked dry-cured meat products has been published by Asefa et al. [41]. Although these two studies examined very different product types (fermented sausages and liver pâté versus dry-cured hams and dry-cured lamb leg) the important genera were established to be *Penicillium*, *Cladosporium* and *Eurotium* in both studies. Other genera previously reported on meat products, as *Alternaria*, *Aspergillus* and *Scopulariopsis* and species belonging to *Mucorales,* were only found sporadically or were absent on the North European meat products. Among frequently reported *Penicillium* species in previous studies, *P. brevicompactum* and *P. solitum* were also isolated frequently from the Danish meat processing plants and *P. chrysogenum*, *P. commune*, *P. cyclopium* and *P. roquefortii* were isolated sporadically. *P. nalgiovense*, which was the dominating species on dry-cured ham in the study of Asefa et al. [41], was not isolated, but the closely related new species *P. milanense* (ined.) was isolated from two of the Danish processing plants. The most frequent species at each processing plant were not the same, for example was *P. palitans* the most frequent *Penicillium* spp. at one of the fermented sausage processing plants, but did not occur at any of the other plants. It can be comprehended that there is a large group of *Penicillium* spp. associated to meat products that are likely to occur, but the group of dominating species varies from processing plant to processing plant. This makes it necessary to examine individual processing plants in order to accurately establish the associated mycobiota and determine if toxinogenic species are present. Several of the isolated *Penicillium* spp. were potential mycotoxin-producers or producers of other secondary metabolites that are unwanted in foods. Indeed, the species *P. brevicompactum / P. bialowiezense* and *P. palitans* were among the prevalent species present in the environment. While *P. brevicompactum / P. bialowiezense* produced the immunosuppressive compound mycophenolic acid, *P. palitans* produced the mycotoxin cyclopiazonic acid (appendix 1). Thus, the conclusion of the first part was that toxinogenic species were part of the potential associated mycobiota of the Danish meat products. Most previous studies of meat products in other regions have revealed toxinogenic species and thus

toxinogenic species must inevitably be considered as part of the associated mycobiota of meat products. This implies that any product with unknown fungal growth could be contaminated with mycotoxins. However, it does not necessarily mean that the toxinogenic species are actually able to produce mycotoxins on meat products or in quantities to be a food safety concern. Whether mycotoxins can be produced on inoculated meat products has been examined in previous studies. Among these, 13 studies [14,68,75,76,88,90-97] detected mycotoxin production by *Aspergillus* or *Penicillium* spp. grown on meat products, while 5 studies [44,65,66,73,74] showed that the studied mycotoxins were not detected at the given conditions and with the analytical methods used. Contradictory observations on whether the mycotoxins penetrated into the meat were also obtained. Such results are not unexpected due to different strains used, different experimental conditions, different meat products and different mycotoxins examined. A weakness of several of the studies was that they were conducted under conditions that do not resemble typical processing conditions.

The aim of the **second part** of the study was to determine if secondary metabolites can be produced and penetrate into the meat on inoculated meat products during realistic processing conditions. An inoculation study was performed, where *P. brevicompactum* was inoculated on dry-cured hams, fermented sausages and liver pâtés (manuscript 2). The products were further processed or stored under conditions typical for Danish meat products, although the fermented sausages were not smoked. Under these conditions, *P. brevicompactum* grew vigorously on all the meat products.

A method for determination of mycophenolic acid (MPA) in meat products was developed. Although this method suffered from the problems typically associated to analytical determination of metabolites in complex matrices as meat products, it was suitable for the purpose of quantitative determination of MPA in fractions of meat products inoculated with *P. brevicompactum*. Improvement of the method may be achieved by modification of the extraction with the specific purpose to lower the ion suppression during MS. A possibility today, which was not available at the time the experimental work was carried out, is the use of ^{13}C-labelled MPA as internal standard that, although expensive, may improve the robustness and reproducibility of the method significantly.

It was shown that *P. brevicompactum* was able to produce MPA in dry-cured ham and liver pâté. This result is an example of the ability of a *Penicillium* sp. to produce secondary metabolites when growing on meat products under fairly realistic processing conditions. An

interesting finding was that MPA was detected even in the centre of dry-cured hams. This implies that removal of the surface mycelium by washing or cutting it away not would be sufficient to avoid contamination of the interior meat with MPA. In contrast, MPA was not detected in any samples of fermented sausages (LOD = 4 μg/kg). Unfortunately, the casings were not analysed and thus it is not possible to distinguish whether MPA was not produced under these conditions or if it was retained on the outside of the casing. However, the results indicate that fermented sausages and the processing conditions used here do not favour MPA production by the strain tested. In the studies of Spotti et al. [75,76] and Iacumin et al. [30] it was shown that OTA was indeed produced on fermented sausages by respectively a *P. verrucosum* strain and the indigenous mycobiota. While Spotti et al. [75,76] detected OTA in the peripheral part of the meat, Iacumin et al. [30] did not detect OTA in the interior meat and neither in casings after brushing and washing of the sausages (LOD =0.1 μg/kg). These opposite results were most likely a consequence of differences in the quantities of OTA produced, but lead to opposite conclusions on the safety of meat products. In general, a conservative approach must be appropriate when assessing whether products are safe. Thus, the conclusion of the second part was that if filamentous fungi were allowed to grow heavily on products, they could potentially produce secondary metabolites that may be found even in the centre of the product. Considering the highest OTA levels measured by Spotti et al. [76] of 20 μg/kg in the peripheral part of the inoculated sausages (a worst case example), then a portion of 50 g sausage per day for a 70 kg person would be enough to reach the provisional tolerable intake, which is not an unrealistic intake. Meat products with uncontrolled mould growth are thus a potential food safety risk. The most critical products are mould-fermented products, if contaminants are not controlled properly, and products with unintended mould growth, where the mould is removed before retail.

To face the problems with potential mycotoxin contamination by filamentous fungi, the meat industry should keep their emphasis on avoiding unintended mould growth on products. Settling of procedures and working routines that prevent spread of conidia or mycelium fragments and thus cross-contamination of products may be a possible means to lower contamination of products. A continuous focus on prevalence of the meat-associated mycobiota, for example by monitoring moulded products, may be useful for identification of variations in conditions or procedures that lead to periodical contamination of products. At the same time, clear-cut procedures for when to discharge moulded products would reduce the

risk of mycotoxin-contaminated products. Regarding products with intended mould growth, i.e. mould-fermented products with added fungal starter culture, the industry may need to improve procedures for assessment and control of contaminating moulds. *P. nordicum* can for example have white conidia and thus be fully camouflaged by the fungal starter culture [60]. Iacumin et al. [30] found contaminating moulds on 75 % of industrially produced products (n = 60) although fungal starter culture was used. Furthermore, OTA was detected in the casings of 58 % of the products. This is of concern for the safety regarding use of fungal starter cultures and illustrates a major drawback; that contaminating mould growth may be camouflaged by the starter culture.

Research can in many ways contribute to enlighten the issue regarding mycotoxin contamination of meat products by filamentous fungi. Further search for toxinogenic species associated to meat products and toxicological evaluations of known and potential new mycotoxins is important as well as studies that can confirm production of the mycotoxins on products *in situ*. It is also important to establish analytical methods for determination of mycotoxins those presences have not yet been examined in meat products as such pioneer examinations can reveal whether the mycotoxins occur at all in meat products and if the levels are significant. Research efforts like these will pave the way for assessment of exposure and hazard of mycotoxins in meat products and eventually for authorities to settle maximum levels for relevant mycotoxins in meat products as well as to establish guidelines for control of moulds and mycotoxins in meat products.

The aim of the **third part** was to gain knowledge about how mycotoxin production is affected by conditions that influence production of mycotoxins, at a cellular level. Combination of starch and lactate in the substrate was found to increase the FB_2 production by *A. niger* synergistically compared to either starch or lactate separately. Buchanan and Ayres [161], El-Gazzar et al. [163] and Luchese and Harrigan [155] similarly observed that lactate could enhance aflatoxin production by *A. parasiticus*. The stimulating effect on aflatoxin production was dependant on the presence of glucose, since other studies did not observe aflatoxin production when lactate was the sole carbon source [164]. This is in congruence with what we observed for the effect of lactate on FB_2 production by *A. niger* in presence or absence of starch. Lactate may have a similar effect on production of mycotoxins in other species. This could be of importance for mould-fermented or mould-susceptible products containing starch or other readily utilisable carbon sources in conjunction with fermentation with lactic acid

bacteria. Indeed, Luchese and Harrigan [155] found that co-culturing of *A. parasiticus* with the lactic acid bacterium, *Lactococcus lactis*, stimulated aflatoxin production. They emphasized to be precautious to avoid foods containing *L. lactis* to being contaminated with aflatoxinogenic moulds. An implication of the results in relation to meat products is that addition of starch to lactate-containing products as fermented sausages could imply a higher risk of FB_2 production during contamination with *A. niger* than expected. It could likewise be the case for aflatoxin production by *A. parasiticus*. Pyruvate has also under certain conditions been shown to stimulate aflatoxin production in *A. parasiticus* [161] and sterigmatocystin production in *A. nidulans* [165] similarly to what was observed for pyruvate on FB_2 production by *A. niger*. For both *A. niger* and *A. parasiticus* it thus seem like lactate as well as the metabolic product pyruvate affect the primary metabolism in a direction that influences the production of FB_2 and aflatoxin respectively.

To reveal the cellular mechanisms affecting FB_2 production by *A. niger* a proteome analysis was conducted. The work was based on the hypothesis that conditions that lead to differences in FB_2 production by *A. niger* would also be reflected in the proteome and thus identification of differently expressed proteins could be used to provide an idea of how FB_2 production is affected at the cellular level. The proteome analysis of *A. niger* showed that levels of several enzymes in the primary metabolism, enzymes involved in anabolic processes and antioxidative proteins correlated with FB_2 production on the media with starch, starch plus lactate and lactate (manuscript 3). These proteins and enzymes were assumed to influence the intracellular levels of acetyl-CoA and regeneration of NADPH and thus the study indicated that high levels of acetyl-CoA and abundance of NADPH correlated with high FB_2 production by *A. niger*. To substantiate this assumption, the intracellular levels of acetyl-CoA and ability to regenerate NADPH should be confirmed by other methods in coming studies. A correlation between availability of precursors and cofactors required for biosynthesis and the actual production is in thread with one of the primary premises used in metabolic engineering to optimise production of metabolites [166]. It has for example been found that the supply of cytosolic NADPH may be critical for the production of penicillin G by *P. chrysogenum* [167]. In several studies of mycotoxin production it have been shown that the influence of substrate nutrients and most changes in environmental conditions were mediated at the level of gene transcription [134-141]. Thus the effect of lactate in combination with starch on FB_2 production in *A. niger* may be expected also to be regulated at the gene level.

One of the outcomes of the proteome analysis was the hypothesis that acetyl-CoA is involved in regulation of FB_2 production in *A. niger* during conditions with high feed / energy state. The influence of acetyl-CoA on regulation may be directly or indirectly. While a direct role of acetyl-CoA could be through modulation of gene transcription or protein activity, indirect roles could be by affecting other regulatory networks. A suggestion for the latter could be that inositol and inositol polyphosphates, that are involved in signal transduction [168], influence secondary metabolite synthesis. We observed an increased level of inositol 1-phosphate synthase during growth of *A. niger* on medium containing starch plus lactate and thus a correlation with high FB_2 production (manuscript 3). Transcription of the *ino1* gene in *A. parasiticus* has also been observed to be induced during a shift from an aflatoxin non-conducive to a conducive medium [140]. As mentioned in manuscript 3 it has been suggested that transcription of the gene encoding inositol 1-phosphate synthase is influenced by acetyl-CoA levels in yeast [169], which could also be the case in *A. niger*. Another potential indirect effect could be that the higher activity of the central metabolism especially the TCA cycle during growth on medium with starch and lactate causes a higher level of oxidative stress as a result of increased oxidative respiration. A correlation between increased levels of reactive oxygen species and increased aflatoxin production has been observed in *A. parasiticus* and involvement of an oxidative stress-related transcription factor in modulation of gene transcription of a gene in the aflatoxin gene cluster has been established [170]. Future studies will tell whether the hypothesis about a direct or indirect role of acetyl-CoA in FB_2 regulation can be confirmed, whether regulation is due to other mechanisms or if the higher FB_2 production on medium with starch and lactate in combination is solely driven by abundance of precursors and/or cofactors. Regardless in what direction these studies goes, the proteome analysis was in this case able to give ideas to how starch and lactate influence the intracellular metabolism of *A. niger* and how this in turn could influence FB_2 production. Along with the availability of more fungal genome sequences, proteome analysis may become an increasingly important tool to explore how environmental factors influence growth and mycotoxin production by filamentous fungi at the cellular level.

To my knowledge the study in manuscript 3 was the first attempt to use proteomics in a study of secondary metabolism in *Aspergillus* and it certainly contributed to the knowledge of FB_2 production in *A. niger*. Such knowledge about regulation of FB_2 production can possibly be used to predict the influence of other substrates on FB_2 production. For example in a study of

A. nidulans, Kim et al. [171] confirmed other studies in that osmoadaptation was associated with increased glycerol production, but they also suggested that other pathways downstream of glycolysis, as the TCA cycle, amino acid biosynthesis and oxidative phosphorylation would be affected by the change in flux towards glycerol production. As a similar response to hyperosmotic conditions is likely to occur in *A. niger*, it can be expected that severe hyperosmotic stress conditions lead to decreased FB_2 production. Another example is the presence of fat in the substrate. If fatty acids are assimilated by *A. niger* and catabolised to acetyl-CoA [172], the combination of starch and fat may be predicted to have a similar effect as starch and lactate. Although these predictions may not hold, they are examples that illustrate how knowledge of regulation of mycotoxins at the cellular level can be used to estimate how changes in product formulation or processing of meat products can influence the mycotoxin production by contaminating moulds. A throughout understanding of the factors that influence growth and mycotoxin production may be of valuable use during hazard analysis of critical control points (HACCP) in the meat production process as well as to avoid unintentional effects of changes in product formulation or the production process. Such preventive measures will limit the risk of mycotoxin contamination of meat products. A further understanding of how mycotoxin production is affected, at the cellular level, may also reveal ideas to new strategies for control of filamentous fungi and mycotoxins in meat products.

Conclusion

The primary objective of this study was to determine if filamentous fungi from meat processing environments can produce toxic secondary metabolites during growth on meat products and to enhance the understanding of conditions that can influence the production. The mycobiota of the examined Danish meat processing plants was diverse and *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Penicillium*, *Phaeoacremonium* and *Phoma* were the main genera isolated. Although fungal spores were prevalent in air and on surfaces in the processing environment, only few products were spoiled by mould growth. It was concluded that fermented sausages were most susceptible to spoilage by *Penicillium* and *Eurotium* species, while liver pâté was expected to be most susceptible to spoilage by *Penicillium* and during the autumn period also *Cladosporium*. It was rationalised that *Penicillium* species were the most important filamentous fungi to be aware of, since many toxinogenic *Penicillium* species were isolated from the processing areas. The most prevalent toxinogenic species were *P. brevicompactum* / *P. bialowiezense* and *P. palitans*, which produce mycophenolic acid and cyclopiazonic acid respectively.

P. brevicompactum was able to produce mycophenolic acid on dry-cured ham and liver pâté in an inoculation experiment in which fairly realistic processing conditions were used. As the products were fractionised, it was possible to determine that mycophenolic acid was present even in the centre of the dry-cured hams and bottom of liver pates. Mycophenolic acid was not detected in fermented sausages and thus was either not produced at levels above the limit of detection at 4 μg/kg or was retained on the surface of the casings. In conclusion, these studies showed that the mycobiota of meat processing environments includes toxinogenic species and that one of these, *P. brevicompactum*, was able to produce mycophenolic acid on meat products like dry-cured ham and liver pâté during realistic processing conditions. Meat products with uncontrolled, unknown mould growth can thus be a potential food safety risk.

The genome sequenced species *Aspergillus niger* was used as model organism to study mycotoxin production using proteome analysis. *A. niger* was just recently found to produce the mycotoxin fumonisin B_2 and thus very little was known of conditions that influence production of fumonisin B_2. Lactate stimulated fumonisin B_2 production by *A. niger* when supplemented in a rich substrate containing yeast extract, nitrate and starch. The production of fumonisin B_4, orlandin, desmethylkotanin and pyranonigrin A was affected by lactate in a similar fashion as fumonisin B_2, while the production of ochratoxin A, ochratoxin alpha,

malformin A, malformin C, kotanin, aurasperone B, tensidol B was not. Identification of proteins within the central metabolism, those levels correlated with fumonisin B_2 production, indicated that the effect of lactate was due to changes in the balance of intracellular metabolites towards high acetyl-CoA levels and abundance of NADPH. Thus, the results indicated that the availability of acetyl-CoA and NADPH has a large influence on the production of fumonisin B_2 by *A. niger*. A further outcome of the proteome analysis was a hypothesis about the role of acetyl-CoA in regulation of fumonisin B_2 production during conditions with a high feed/energy state. The use of proteome analysis did here enhance the understanding of how starch and lactate influence fumonisin B_2 production by *A. niger* and may be a valuable tool in future studies of mycotoxin production by filamentous fungi.

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Appendix 1

Sørensen LM, Frisvad JC, Jacobsen T, Koch AG, Nielsen PV: Toxic Secondary Metabolites of *Penicillium* strains from Meat Processing Plants, poster presented at International dry-cured meat congress, Oslo, June 2006.

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danish meat association
DMRI
DTU

Toxic Secondary Metabolites of *Penicillium* strains from Meat Processing Plants

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Aim

The aim was to explore how growth and mycotoxin production by *Penicillium brevicompactum*, *P. bialowiezense*, *P. commune* and *P. palitans* was influenced by substrate compositions typical for meat products.

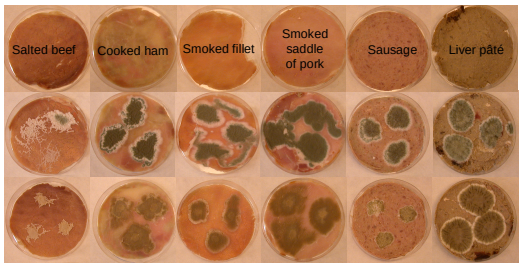
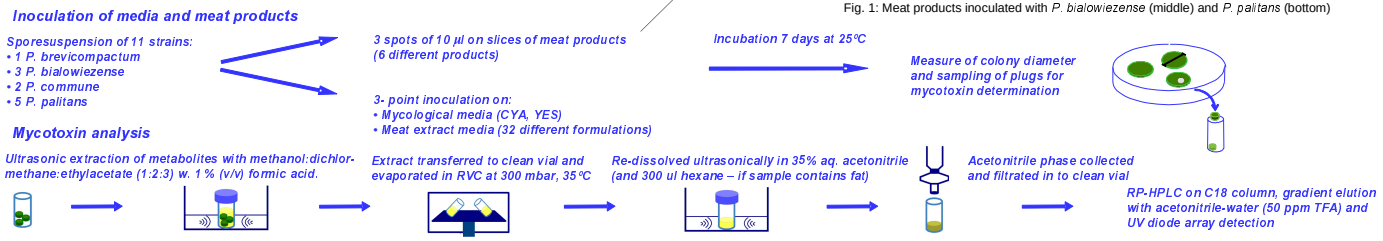


Fig. 1: Meat products inoculated with *P. bialowiezense* (middle) and *P. palitans* (bottom)

Methods



Results

The species studied produce many different secondary metabolites; at least 16 were identified. We focus on two metabolites, which are known to be toxic. Mycophenolic acid (MPA) is an immunosuppressive and is produced by *Penicillium brevicompactum* and its sibling species *P. bialowiezense*. Cyclopiazonic acid (CPA) is a neurotoxin and is produced by *P. commune* and *P. palitans*.

The amount of MPA and CPA was assessed quantitatively, and showed high variation between strains.

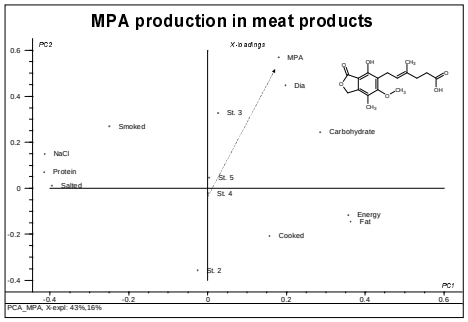


Fig. 2: PCA of colony diameter and MPA production of *P. brevicompactum* (st. 2) and *P. bialowiezense* (st. 3, 4 and 5) on meat products together with product characteristics.

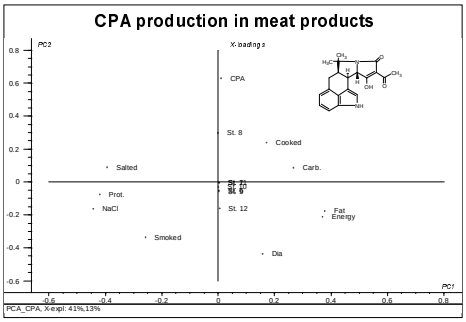


Fig. 3: PCA of colony diameter and CPA production of *P. commune* (st. 10 and 11) and *P. palitans* (st. 6, 7, 8, 9 and 12) on meat products together with product characteristics. St. 6, 7, 8, 9 refer to strain numbers.

Mycotoxin production on meat products

P. brevicompactum and *P. bialowiezense* produced mycophenolic acid in all samples of meat products (n=24). Production correlated with colony diameter and carbohydrate content, while the other product characteristics had little influence (fig. 2). *P. commune* and *P. palitans* produced cyclopiazonic acid in 98 % of the meat product samples (n = 42). Colony diameter correlated negatively with CPA produced. Growth on cooked or smoked products influenced CPA production a little (fig. 3).

Influence of NaCl

P. brevicompactum and *P. bialowiezense* are halophilic species, which is probably the reason why MPA production not was much influenced by a change from 1 to 5 % NaCl (fig. 4). *P. commune* and *P. palitans* produced less CPA at 5% than at 1 % NaCl (fig. 5).

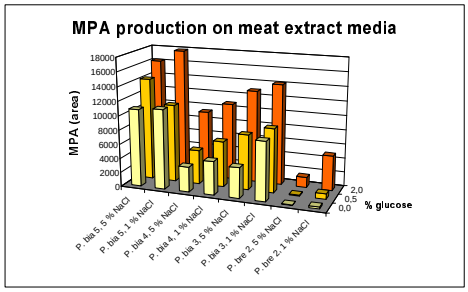


Fig. 4: Influence of 1% and 5 % NaCl and 0 – 2 % glucose on MPA production of *P. brevicompactum* and *P. bialowiezense* on meat extract media.

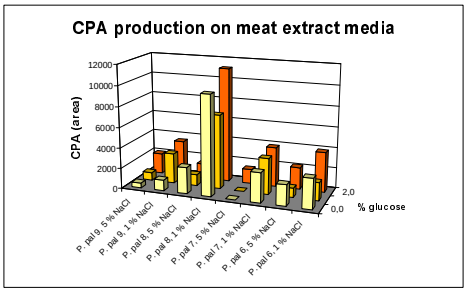


Fig. 5: Influence of 1% and 5 % NaCl and 0 – 2 % glucose on CPA production of *P. palitans* on meat extract media.

Influence of glucose

Addition of glucose to meat extract media had a stimulating effect on especially MPA production (fig. 4).

Influence of fat

Both growth and CPA production of *P. commune* and *P. palitans* was stimulated by fat on meat extract media (fig. 7), which agrees with the lipolytic capabilities of these strains. Fat had no influence on MPA production (fig. 6).

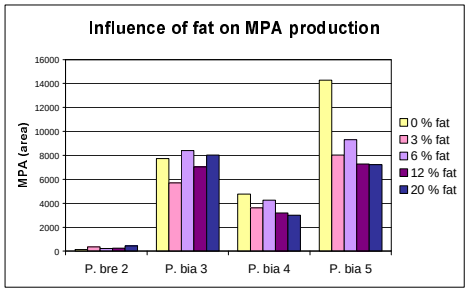


Fig. 6: MPA production of *P. brevicompactum* and *P. bialowiezense* on meat extract media (0,5 % glucose, 5 % NaCl) with increasing concentration of fat.

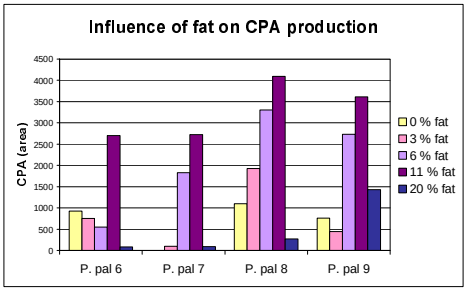


Fig. 7: CPA production of *P. palitans* on meat extract media (0,5 % glucose, 5 % NaCl) with increasing concentration of fat.

Conclusion

The fungi in this study were clearly able to produce mycotoxins on meat products, so fungal growth on a product must be considered as a high risk of mycotoxin contamination. Strain variation is however high. Product composition is important for MPA and CPA production. Meat products with high salt and/or carbohydrate content may be susceptible to MPA contamination, while CPA could be critical for fatty products. More metabolites were detected on meat products compared to meat extract media. Under these experimental conditions, 25°C and normal atmosphere, liver pâté was an especially good substrate for production of secondary metabolites. This probably reflects the high carbohydrate and fat content, and availability of many micro-nutrients.