International Commission on Food Mycology

Workshop 2013

Food Mycology in a Globalized World – Challenges and Solutions to the Safety of Food

Freising, Germany - 3 – 5 June, 2013
International Commission on Food Mycology

The commission is a COMCOF (Commissions, Committees and Federations) of the International Union of Microbiological Societies (IUMS) and established in 1990.

The aims of the Commission are:

- to improve and standardise methods for isolation, enumeration and identification of fungi in foods;
- to promote studies of the mycological ecology of foods and commodities;
- to interact with regulatory bodies, both national and international, concerning standards for mycological quality in foods and commodities;
- to support regional initiatives in this area. The Commission further aims to extend understanding of the principles and methodology of food mycology in the scientific community by publishing its findings, and by sponsoring meetings, specialist workshops, courses and sessions dealing with aspects of its work.

Venue:
Freising is a 50,000 citizen community situated 40 km north east from the city of Munich, which can be reached by train in 20 min. Munich Airport is close by with a direct bus connection.

The city is well known for its rich ecclesiastic history as well as for its importance as a centre of food science and technology as well as beer brewing. The Freising Cathedral (built 1205) it is one of the two home churches to the archbishop of Munich and Freising, one of which was Cardinal Josef Ratzinger, who was elected pope Benedikt XVI in 2005. The workshop will take place at the “Kardinal Döpfner Haus”, a venue within the premises of the cathedral with lecture rooms and accommodation for participants.

Sponsors

IUMS
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The seventh International Foodmycology workshop is organized by Ludwig Niessen (Freising) and Rob Samson (Utrecht).
ICFM 2013 AbstrACts

PROGRAMME ICFM 2013

Sunday, 2. June 2013

16.00 Registration

18.00 Get together at “Klause” bar (drinks and finger food)

Monday, 3. June 2013

08.00–09.30 Registration continued

09.30–09.45 Opening of the workshop and welcome addresses

Session 1: Taxonomy and biodiversity of food borne fungi (Chairperson J.C. Frisvad)

09.45–10.10 Robert A. Samson: New taxonomies and nomenclature of foodborne fungi of the Trichocomaceae

10.10–10.35 Jos Houbraken: Taxonomic changes of food-borne Aspergillus and Penicillium species

10.35–11.00 Marta Taniwaki: Biodiversity of toxigenic Aspergillus species in Brazil: occurrence, polyphasic taxonomy and distribution

11.00-11.30 Coffee break

11.30–11.55 Sofia Chulze: Characterization of Aspergillus section Flavi in soils from new peanut growing areas of Argentina

11.55–12.20 Soňa Felšöciová: Occurrence of penicilli on grapes grown in selected Slovak regions

12.30–13.30 Lunch break at Kardinal-Döpfner-Haus

Session 1, continued

13.30–13.55 Endang S. Rahayu: Ochratoxigenic black aspergilli isolated from dried agricultural products in Yogyakarta, Indonesia

13.55–14.20 Tom Gräfenhan: Differences in toxigenicity and growth of Penicillium verrucosum strains on cereals and their propensity to form ochratoxins

Session 2: Spoilage problems and prevention of food borne moulds and yeasts (Chairperson J. Dijksterhuis)

14.25–14.50 Emilia Rico: Occurrence of heat-resistant mold ascospores in the processing environment: Methodology, prevention and elimination

14.50–15.15 Deborah Waters: Identification of the fungal microflora of coffee beans from different origins and evaluation of lactic acid bacteria fungal antagonism

15.15–15.40 Claudia Axel: Lactic acid bacteria producing anti-fungal compounds: biopreservation in cereal products

15.40–16.00 Coffee break

Session 3: Mycotoxins and other fungal extrolites in food and beverages (Chairperson Sofia Chulze)

16.05–16.30 Javier Cabañes: Are there nonochratoxigenic wild strains of Aspergillus carbonarius?

16.30–16.55 Giancarlo Perrone: Monitoring ochratoxin A risk during the seasoning of salami

17.00–18.30 Poster session

19.00 Dinner at Kardinal-Döpfner-Haus
Tuesday, 4. June 2013

Session 3, continued

09.00–09.25 Elisabeth Fredlund: Fungi and mycotoxins in goji berries, oil seeds and walnuts on the Swedish retail market
09.25–09.50 Gemma Castella: PKS diversity in Aspergillus carbonarius
09.50–10.15 Maria-Laura Ramirez: Fusarium graminearum and deoxynivalenol in wheat spikes, grains and flour in Argentina: effect on food safety and quality of wheat grains and by-products
10.15–10.40 Jens C. Frisvad: Mycotoxins and fungal drugs in foods

10.40 - 10.55 Coffee break
11.00–11.25 Ulf Thrane: Food colorants from fungi
11.25–11.50 Rolf Geisen: Chlorogenic acid, a metabolite identified in tomato fruits by a metabolomics approach, is inhibitory against the biosynthesis of alternariol by Alternaria alternata
11.50–12.15 Yang Liu: Occurrence of the Alternaria toxins tentoxin, dihydrotentoxin and isotentoxin in foods

12.15–13.15 Lunch break at Kardinal-Döpfner-Haus

Session 4: Influence of environmental factors on the physiology of foodborne fungi (Chairperson Rob Samson)

13.30–13.55 Jan Dijksterhuis: Fungal spores as vehicles for dispersion in space and time
13.55–14.20 Naresh Magan: Environmental stress affects fungal colonisation and metabolomic profiles in stored wheat and maize
14.20–14.45 Angel Medina-Vaya: Integrating ecophysiology and gene expression data to understand Fusarium verticillioides production of fumonisins
14.45–15.10 Alicia Rodriguez: Salt (NaCl) concentration affects growth and temporal ochratoxin A production by Penicillium nordicum strains on a ham-based medium
15.10–15.35 Timon Wyatt: Changes in the physical state of the cytoplasm and accumulation of compatible solutes are related to the stress resistance of heat-resistant ascospores

15.35–15.50 Coffee break

15.50–16.15 Dominic Stoll: Mutual regulation of the biosynthesis of ochratoxin A and citrinin in Penicillium verrucosum as an adaptation to different habitats
16.15–16.40 Frank Segers: Comparison of survival of Cladosporium and Penicillium species during different developmental stages under low water activity
16.40–17.05 Timon Wyatt: Mannitol is essential for the development of stress resistant ascospores in Neoartomya fischeri

17.10–19.00 ICFM Committee meeting (closed meeting)
20.00 Dinner at Bavarian Biergarden
Wednesday, 5. June 2013

Session 5: Open session (Chairperson Ludwig Niessen)

09.00–09.25 Carla Denschlag: A real-time multiplex loop-mediated isothermal amplification (LAMP) assay for simultaneous detection of important trichothecene producing Fusarium spp. in wheat

09.25–09.50 Jie Luo: Development and application of a loop-mediated isothermal amplification assays for rapid identification and detection of aflatoxicigenic moulds in food samples

09.50–10.15 Yang Xu: Ochratoxin A mimotopes from an evolved phage library and its application in immunoassay

10.15–10.40 Su-lin Leong: Genome and transcriptome of Xeromyces bisporus, a model(?) xerophile

10.40–11.05 Richard van Leeuwen: Novel insights in the mode of action of polyene antibiotics

11.15–11.45 Coffee break

11.45–12.15 Final discussions, closing of the workshop

12.30 Lunch at Kardinal-Döpfner-Haus, Checkout

Posters (Poster session on Monday, 3 June, 17.00–18.30)

Elettra Berni: Heat-resistance of Hamigera and Thermoascus strains isolated from pasteurized fruit-based foods

Aidan Coffey: Biological control of Mycophaerella graminicola, causitive agent of Septoria tritici blotch (STB) of wheat, using antifungal Lactobacillus brevis

Francesca Degola: Mycotoxin biocontrol: two examples of intra- and inter-specific biocompetition

Samart Dorn-In: Reconstruction of the original fungal flora of heat-processed meat products by PCR-SSCP

Beatriz Iamanaka: Aspergillus section Flavi and aflatoxins in sugarcane juice and dried yeast

Zuzana Mašková: Toxigenicity of Alternaria spp. isolated from grapes of Slovak origin

Julia Matthes: Fusarium toxins in potatoes and potato products

Andrea Patriarca: Water activity and temperature effects on mycotoxin production by Alternaria arborescens

Maria Helena Pelegrinelli-Fungaro: A survey of genes for ochratoxin and fumonisin biosynthesis to select both mycotoxin nonproducers strains of Aspergillus niger

Giancarlo Perrone: Influence of varying temperature and water activity on fungal growth, production of AFB1, and expression of aflatoxin biosynthesis genes in Aspergillus flavus on almond medium

Dojin Ryu: Determination of ochratoxin A in breakfast cereals and snacks from the United States by high performance liquid chromatography with fluorescence detection

Daniele Sartori: Proteomic analysis of a mutant strain of Aspergillus westerdijkiae for production of ochratoxin A

Daniele Sartori: Genetic and mycotoxin data analyses reveal the contamination of Brazil nuts by Aspergillus pseudonomius

Markus Schmidt-Heydt: Fungicides effectively used for growth inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species

Dana Tancinova: Potential producers of ochratoxin A on grapes of Slovak origin

Marta Taniwaki: Fungi producing fumonisin B1 in Brazil nuts

Marina Venturini-Copetti: Fungal spoilage in frozen chicken nuggets

Phil Voysey: Mapping the heat resistance of fungi under conditions of pH and sugar concentration associated with beverages
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NEW TAXONOMIES AND NOMENCLATURE OF FOODBORNE FUNGI OF THE 
Trichocomaceae

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The taxonomy of food-borne fungi has changed since the introduction of
the polyphasic taxonomic approach using phenotypic, biochemical and
molecular data. Phylogenetic studies have revealed new insights in the
relation and delimitation of genera in the Trichocomaceae, a family which
contains many genera important in food mycology. These studies have
shown the relationship between the two important genera Penicillium and
Aspergillus. A major conclusion is that the species belonging to Penicillium
subgenus Biverticillium are distantly related from Penicillium and that this
group of fungi needs to be accommodated in a separate genus Talaromyces.
The rational of selecting this name will be discussed in view of the recently
published International Code of Nomenclature for Algae, Fungi and Plants.
This Code made an end to the dual fungal nomenclature giving anamorph
names the same priority as teleomorph names. The single nomenclature is
discussed in Penicillium, Aspergillus and a proposal is presented to select
single names in the remaining genera of the food-borne Trichocomaceae.

Taxonomic changes of food-borne Aspergillus and Penicillium
species

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Identification of a species is an important step in biological research. A
correct name is vital for optimal communication, and is often the link
between studies of various fields. It is therefore important that the taxonomy
is clear and stable. Ideally, identification should be unequivocal, accurate,
simple and immutable. In the last decade, new insights have resulted that
certain well-known species belonging to Aspergillus or Penicillium appear
to be species complexes. This might lead (initially) to confusion; however,
a correct identification has a function: certain species of these complexes
have unique properties such as higher resistance to certain antifungals,
production of different mycotoxins and/or have unique enzyme profiles.
In this paper, an overview of recent name changes and developments in
the taxonomy of food borne Aspergillus and Penicillium species is given.
For example, P. chrysogenum proved to be a complex of five species and
two of those species (P. rubens and P. chrysogenum) appear to be commonly
occurring. The taxonomy of species belonging to Aspergillus section Nigri is
revisited and A. awamori is considered a doubtful name.
**Biodiversity of toxigenic *Aspergillus* species in Brazil: occurrence, polyphasic taxonomy and distribution**

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Brazil has an underexplored biodiversity of toxigenic species of fungi including *Aspergillus* species that are among the most important mycotoxin-producing fungi in foods. The objectives of the present work are to study the distribution of toxigenic species of *Aspergillus* in several foods in different regions of Brazil and generate a data base with all phenotypic, genotypic and phylogenetic information. Bean or nut samples were surface disinfected with 0.4% chlorine solution for 2 min, then 33 - 50 beans from each sample were plated directly onto Dichloran 18% Glycerol agar, and incubated at 25°C for 5 days. For powder and flocculated samples the dilution plate technique was used on the same culture medium and incubation condition. After incubation, plates were examined and all fungal species were first isolated onto Czapek Yeast extract agar (CYA) plates for further identification. Up to date, a total of 9,940 potentially toxigenic species of *Aspergillus* have been isolated from coffee, cocoa, brazil nuts and dried fruits (dates, sultanas, figs and prunes). *Aspergillus* section *Flavi* has predominated with 5,706 isolates, followed by *Aspergillus* section *Nigri* with 3,666 and *Aspergillus* section *Circumdati* with 568 isolates. From these, 0.9%, 0.1%, 20% and 79% of *Aspergillus* section *Flavi*; 21%, 13%, 10% and 56% of *Aspergillus* section *Nigri* and 90%, 6%, 3% and 1% of *Aspergillus*. section *Circumdati* have been isolated from coffee, dried fruits, cocoa and brazil nut samples, respectively. *Aspergillus* section *Nigri* appeared in considerable numbers on all substrates, confirming the predominance of these as food contaminants. The samples of Brazil nuts contributed with the largest number of isolates, especially because of the biodiversity of Amazon rain forest.

**Characterization of *Aspergillus* section *Flavi* in soils from new peanut growing areas of Argentina**

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Peanuts is an economically important crop in Argentina, with Cordoba Province (main growing peanut region) accounting for approximately 98% of the total production. Diverse factors such as soil fungal pathogens, climatic conditions and soil degradation can explain the exploration of new growing areas in others provinces or in marginal areas of the main peanut growing region. The aim of the present study were to extend the knowledge of the biodiversity of *Aspergillus* section *Flavi* soil populations from new peanuts growing areas and to compare these populations with population isolated from the traditional peanuts growing region. Soil samples were collected from Formosa Province, La Pampa Province and south of Cordoba Province during 2008 harvest season. Total mycobiota and *Aspergillus* section *Flavi* was done by surface spread method on DRBC and DG18 media. Four hundred and thirty isolates belonging to *Aspergillus* section *Flavi* were characterized by morphology, sclerotia production and mycotoxin production (aflatoxin and cyclopiazonic acid production). The
mean values of total mycobiota and *Aspergillus* section *Flavi* were $3.6 \times 10^4$ and 319 UFC/g, respectively. Out of 430 strains isolated within the *Aspergillus* section *Flavi* 90 % were identified as *A. flavus*, 6 % as *A. parasiticus* and 4 % were remained unidentified using morphological markers. These strains were further identified as *A. caelatus* using calmodulin gene sequences. Among the *A. flavus* isolated 88 % were L strains, 3% were S strains and 10 % were not able to produce sclerotia. Seventy one percent of *A. flavus* were aflatoxin producers and 81 % were cyclopiazonic acid producers. A relatively large proportion of *A. flavus* strains (n= 111, 29 %) isolates were not able to produce aflatoxin. Molecular analysis of *omt-A*, *ver-1*, *nor-1* and *afl-R* genes of 34 strains of non aflatoxigenic *A. flavus* showed that 19 strains had absence of 1, 2 or 3 of the genes analyzed. Only 1 strain showed absence of all 4 genes studied. There were no significant differences in the mean level of AFB$_1$ production among strains isolated from the different new areas of peanut cultivation. The levels of toxin produced were lower than those produced by strains isolated from areas with long term history of peanut cultivation. Principal component analysis showed that fields with recent history of peanut cultivation are closely related with the isolates belonging to *A. flavus* L phenotype, producers of low aflatoxin levels, while typical groundnut regions are closely related with high percentages of *A. parasiticus* and *A. flavus* S phenotype, producers of higher aflatoxin levels.

**Occurrence of Penicilli on grapes grown in selected Slovak regions**

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The concern about filamentous fungi in the vineyard has traditionally been linked to spoilage of grapes due to fungal growth. Saprobic fungi can cause rot in grapes and in addition produce mycotoxins. One of the genera responsible for it is *Penicillium*. The aim of this study was to monitor the mycobiota in Slovak origin grapes, with focus on genera *Penicillium* in the year 2011. The potentially toxigenic species were *in vitro* tested by TLC for the ability to produce selected mycotoxins. Altogether twenty samples of wine grapes (white – 16 samples, red – 4 samples) from the sixteen vineyards (sub regions) were collected during harvesting in: Central Slovak wine region, South Slovak wine region, Lesser Carpathian wine region and Nitra wine region. Fifty wine grape berries per bunch (7-8 berries per plate) which showed no symptoms were randomly selected, directly plated onto Dichloran Rose Bengal Chloramphenicol agar medium and incubated for 5 to 7 days at 25 ± 1°C in the dark. Of these samples were identified 22 genera, 90 % of samples were colonised by the genus *Penicillium*. Two hundred and sixty seven *Penicillium* colonies were isolated and identified to 8 species, namely *P. aurantiogriseum*, *P. citrinum*, *P. crustosum*, *P. expansum*, *P. chrysogenum*, *P. polonicum*, *P. thomii* and *P. sp*. The highest level of colonisation was found for *Penicillium chrysogenum* (35 % of the samples) and this species also was dominant with 45 % of relative density. Five potentially toxigenic species were tested for their toxigenic ability. Out of 33 strains, 94 % produced at least one mycotoxin.

The study was supported by the project: Development of International Cooperation for the Purpose of the Transfer and Implementation of Research and Development in Educational Programs conducted by the Operational Program: Education, ITMS code: 26110230085. The research was financially supported by the project KEGA-005SPU-4/2011.
**OCHRATOXICGENIC BLACK ASPERGILLI ISOLATED FROM DRIED AGRICULTURAL PRODUCTS IN YOGYAKARTA, INDONESIA**

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Black aspergilli, potential producers of ochratoxin A (OTA), were the predominant fungi in fermented cocoa bean, coffee bean, and dried cassava in Yogyakarta. Identification of black aspergilli at species level will be useful to make clear link between OTA contamination of food products and the toxin producer. The objective of this study is to identify the species of the black aspergilli producing OTA which contaminated dried agriculture products. In this study, 16 isolates were obtained, and 4 isolates among of them (YAC-9, YAK-6, YAK-12, YAG-2) were found as OTA producing-strains, with the highest OTA found on YES solid medium of 57.68 ppb. Based on morphological characters, 16 isolates can be grouped into 4 species, after confirmation by molecular data based on a PCR method, the groups were identified as *A. carbonarius*, *A. niger*, *A. tubingensis*, and *A. aculeatus*. OTA producing-strains were identified as *A. carbonarius* and *A. niger*, while strains of *A. tubingensis* and *A. aculeatus* were found to be non OTA producers.

**DIFFERENCES IN TOXIGENICITY AND GROWTH OF PENICILLIUM VERRUCOSUM STRAINS ON CEREALS AND THEIR PROPENSITY TO FORM OCHRATOXINS**

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Twenty six strains of *Penicillium verrucosum* (Pv) were isolated from individual seeds randomly selected from 19 different shipments of western Canadian oats or barley. These isolates were identified using micro- and macro-morphological characters on a number of different media including dichloran yeast sucrose glycerol agar (DYSG) and coconut cream agar (CCA). On DYSG, all Pv isolates formed colonies with a distinctive red-brown to terracotta brown reverse. In the first part of the study, twelve of the Pv isolates were selected for inoculation of sterilized rice (19% moisture content) with agar plugs colonized by individual strains. After 4 weeks of incubation at 25°C, colonized grains of rice were transferred to single CCA plates. The remainder of the rice was used for the quantitation of mycotoxins, namely citrinin, ochratoxin A and B. After incubation of inoculated CCA plates in the dark, fluorescence colouring of reverse colonies was documented for correlation with the propensity to form ochratoxin A (OTA) in culture. Under UV light, the intensity of the blue-turquoise fluorescence of the CCA plates did not mirror the level of actual OTA production by most of the Pv strains tested. In part 2 of the study, substrate specificity of ochratoxin production was tested on replicates of sterilized oats and wheat (19.7% moisture content) by inoculation with 26 toxigenic or non-toxigenic Pv isolates. After 3 months of incubation at 20°C, each sample was analysed for ochratoxins and fungal biomass was determined (ergosterol analysis). Comparing levels of ochratoxin A and B contamination on inoculated wheat and oats, each Pv isolate performed similar on both substrates. On wheat, however, similar amounts of ochratoxins were produced by less than 20% of the fungal biomass compared to oats. These observations were made consistently for all 26 Pv strains tested. The mode of colonization of
wheat kernels by Pb was studied using scanning electron microscopy (SEM). Kernels fractured longitudinally transverse through the crease showed mycelial growth in the endosperm tissue and around the germ.
Occurrence of heat-resistant mold ascospores in the processing environment: Methodology, prevention and elimination

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Heat-processed beverages can be spoiled by heat-resistant molds (HRM). The HRM ascospores can not only survive the heat treatment given to these beverages but also be activated and grow during storage. It is known that HRM ascospores are found in ingredients (sweeteners, juice concentrates, juice purees, vitamin powders, energy powders, maltodrexitin, pectin, etc.) and in empty PET bottles. There is not much information on the role that the processing environment plays on the spoilage of these beverages. This study was undertaken to determine the extent of the contamination by HRM ascospores of the processing environment. More than 1,000 environmental samples were tested for HRM ascospores. Environmental samples were collected at nine beverage processing plants. Large surface areas were taken using sterile sponges containing neutralizing buffer. A bottle of 1% peptone buffer was added to the bag and the sponge was stomached for two minutes. Samples were filtered. The filter was washed and transferred to a sterile bag. Aliquots of peptone buffer (10 ml) were added to the bag and contents were heat shocked at 75 ºC for 30 min. Bags were rapidly cooled down. Aliquots were plated using malt extract agar (MEA) with antibiotics. Plates were incubated at 30 ºC for up to 3 weeks. The areas with the highest counts of ascospores were the palletizer (more than 16% positive) and the stretch wrap around the empty bottle pallets (more than 10%). More than 3-5% of the samples taken from the depalletizer, slip sheets in between layers of empty bottles, the airveyor, the filler and cooling tunnel areas were also contaminated by ascospores. The most common HRM isolated was B. spectabilis followed by Neosartorya fischeri, B. nivea and Eurotium amstelodami. In conclusion, the processing environment could contribute to a small rate of spoilage of heat-processed beverages.

Identification of the fungal microflora of coffee beans from different origins and evaluation of lactic acid bacteria fungal antagonism

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This work aimed to identify indigenous fungi from commercial green Coffea arabica and Coffea robusta coffee beans, and to evaluate lactic acid bacteria (LAB) for their anti-fungal effects against the isolated strains. Fungi were isolated using standard techniques and identified by DNA-sequencing and morphological analyses. Fungal isolates generally belonged to Aspergillus, Penicillium, Fusarium, Rhizopus, and Mucor genera. LAB or crude mixtures of LAB-CFS (cell free supernatant) were investigated to control/inhibit fungal growth of coffee bean isolates. The in vitro antagonistic LAB plate test was done on bacterial agar containing pre-cultured LAB spots with the subsequent overlay of fungal spore-containing agar. The plates were then
incubated aerobically at 25°C for 7 days. The LAB-CFS test was done by incorporating CFS-agar into PDA agar, with subsequent application of fungal spore-containing agar. The plates were incubated as for the LAB plate test. The efficacy of the in vitro approaches were evaluated based on visual determination of fungal growth and measurement of fungal-free halos surrounding LAB spots in the LAB plate tests, or measuring % plate covered by fungal mycelia in the LAB-CFS plate tests. Additionally, selected antifungal LAB strains; Lactobacillus amylovorus FST 2.11, L. reuteri R29, L. reuteri hhp and L. brevis R2Δ, and their negative counterparts (L. amylovorus DSM20552, L. reuteri L1105, L. reuteri M13 and L. brevis DSM20053, respectively) were also tested in situ. The use of LAB was successful in the inhibition of a broad range of fungal species from diverse geographical locations.

**Lactic Acid Bacteria Producing Anti-Fungal Compounds: Bio-Preservation in Cereal Products**

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Fungal food spoilage plays a pivotal role in the deterioration of food systems with potential production of harmful metabolites. Additionally, fungal spoilage pathogens lead to reduced quality, safety and hygiene of foodstuffs, resulting in major economic problems throughout the world. Nowadays, food spoilage can be prevented using physical and chemical methods, but no efficient strategy has been proposed thus far to reduce microbial growth thereby ensuring public health protection. We propose the use of lactic acid bacteria (LAB) as natural preservatives. The protection of food products using LAB is mainly due to the production of antifungal compounds such as; carboxylic acids, fatty acids, ethanol, carbon dioxide, hydrogen peroxide and bacteriocins. In addition to this, LAB can also positively contribute to the flavour, texture and nutritional value of food products. This presentation focuses on the use of LAB for food preservation in bread and malt cereal products as model food systems. However, their biopreservation function can also be extended to other food and feed industry applications.
SESSION 3 - MYCOTOXINS AND MYCOTOXIGENIC FUNGI

Chairperson: Naresh Magan

ARE THERE NON-OCHRATOXIGENIC WILD STRAINS OF ASPERGILLUS CARBONARIUS?

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Ochratoxin A (OTA) is a potent nephrotoxin which may contaminate mainly cereals and their products, but is also found in a variety of common foods and beverages (e.g. bread, chocolate, coffee, wine). This mycotoxin is produced by several species of Penicillium and Aspergillus, although few of them are known to contaminate foods with OTA. The contamination of foods by OTA is an important food safety concern for consumers. In the last decade, it has been clearly demonstrated that some species belonging to the black aspergilli, specially A. carbonarius, are the responsible sources of OTA in food commodities such as wine, grapes or dried vine fruits from main viticultural regions worldwide. OTA production consistency varies in the reported OTA producers in black aspergilli. Although not all the strains belonging to an ochratoxigenic species are necessarily producers, in the case of A. carbonarius the percentages of ochratoxigenic isolates reported in this species are usually high and reach 100% in some studies confirming its consistency in producing this mycotoxin. In fact, non-OTA producing strains morphologically identified as A. carbonarius involved in the OTA contamination of grapes were subsequently correctly identified and described as a new species named A. ibericus. In this meeting the results obtained in the characterization of a wide variety of A. carbonarius strains isolated from grapes in our laboratory will be presented. All the A. carbonarius strains studied were unambiguously identified by morphology and genotypic methods. The identification of the strains was confirmed by ITS-5.8S rRNA, β-tubulin and calmodulin gene sequencing.

MONITORING OCHRATOXIN A RISK DURING THE SEASONING OF SALAMI

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Fungi have an important role in the production of dry-cured meat products, especially during the seasoning period of both industrially and handmade salami, when their surface is quickly colonized by a composite mycobiota. The most frequently isolated genera are Aspergillus, Eurotium, and Penicillium, with Penicillium species being predominant due to their use for the improvement of organoleptic characteristics and for preventing the growth of pathogenic, toxigenic or spoilage fungi. The main occurring Penicillium species are P. nalgiovense, P. olsonii, P. brevicompactum, P. chrysogenum and a not yet well described species referred to as “P. milanense”. The occurrence of ochratoxin A (OTA) producing species, such as P. nordicum, has also been reported. Therefore the development of rapid diagnostic methods for its monitoring during the curing phase of meat is needed in order to reduce OTA risk in cured meats or hams. Within the Ministerial project “SAFE-MEAT”, we identified and monitored fungal species on salami surface and in the air occurring in Dodaro’s salami plant (Calabria, Italy). P. nalgiovense and “P. milanense” resulted as the major
indigenous starters in Dodaro’s plant. In addition innovative molecular and olfactometric methods have been developed to assess OTA risk in cured meat. A Reverse Transcriptase PCR (qRT-PCR) assay was set up to quantify OTAtranscripts in P. nordicum grown under conducive conditions; results were positively correlated to OTA production. Moreover, we have applied a MOS-based electronic nose to a meat based medium inoculated with OTA producing and non-OTA producing Penicillium species to assess specific volatile production patterns (VPPs) of OTA producers. Both methods are now in progress for their applicability in assessing OTA contamination in the cured meat supply chain.

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FUNGI AND MYCOTOXINS IN GOJI BERRIES, OIL SEEDS AND WALNUTS ON THE SWEDISH RETAIL MARKET

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The foods most commonly associated with the occurrence of mycotoxins include dried fruits, nuts and spices. Limit values have been set within the European legislative framework [(EG) 1881/2006] for the most important mycotoxins like aflatoxins, ochratoxin A, fumonisins, patulin, zearalenone and some trichotheccenes in identified food categories. Dried nuts are very susceptible to infection by moulds, where only a slight increase in moisture content gives an opportunity for the fungi to grow. Aspergillus flavus is the main producer of aflatoxins in nuts but A. parasiticus and A. nomius may also occur. Small oil seeds have also been reported to contain mycotoxins, with A. flavus and aflatoxins as the main threat. However, other toxigenic molds also occur. Preliminary results from analysis of wolf berries indicate that this type of product often contains high levels of moulds but less is known on the presence of mycotoxins. The objectives of this study were: (i) to increase the knowledge of the mycobiota of wolf berries and small oil seeds with focus on mycotoxin producing species, (ii) to screen samples of walnuts, wolf berries and small oil seeds for the presence of aflatoxins and ochratoxins A as a basis for future risk management measures, and (iii) to evaluate the use of ELISA analysis for quantification of aflatoxins and ochratoxin A in samples of walnut, small oil seeds and wolf berries. Approximately 10 samples from each product category (wolf berries, sesame seeds, pumpkin seeds, sunflower seeds, and walnuts) were analyzed with ELISA and traditional cultivation techniques. Aflatoxigenic species were isolated from 80% of the walnut samples, 60% of the wolf berry samples, and 30% of the oil seed samples. However, only one sample of walnuts contained more than 4 ppb aflatoxins. Potential producers of ochratoxin A were isolated from most samples and 70% of the walnut samples contained more than 5ppb of ochratoxin A.

POLYKETIDE SYNTHASE DIVERSITY IN ASPERGILLUS CARBONARIUS

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At present, wine is considered as the second major source of ochratoxin A (OTA), being OTA contamination of grapes a serious health and economic problem in vine-growing regions. The main source of OTA contamination of grapes is Aspergillus carbonarius and at lesser extent several members of the A. niger aggregate. A. carbonarius consistently produces large amounts of
ochratoxin A (OTA), and the reported percentage of OTA-producing strains is very high. To date, there is limited information about the genes involved in the OTA biosynthesis in A. carbonarius. Several polyketide synthase genes (PKS) have been described and had been suggested to be involved in the first steps of OTA biosynthetic pathway, but the role of these PKS genes remains unclear. Recently, a gene encoding a nonribosomal peptide synthase has been demonstrated to be involved in OTA biosynthetic pathway of A. carbonarius. The aim of the present study was to amplify and sequence five kethosynthase (KS) domains from PKS genes previously described in A. carbonarius. A wide variety of A. carbonarius strains isolated from grapes in our laboratory was analyzed. We detected the five KS domains in all strains and analyses of the sequences revealed some genetic diversity among them.

**Fusarium graminearum and Deoxynivalenol in Wheat Spikes, Grains and Flour in Argentina: Effect on Food Safety and Quality of Wheat Grains and By-Products**

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Fusarium graminearum sensu stricto is the predominant causal agent of Fusarium Head Blight (FHB) in Argentina, resulting in economic losses through reduction in grain yield, quality and accumulation of mycotoxins mainly trichothecenes such as deoxynivalenol (DON). During the harvest season 2012/2013 an epidemic of FHB occurred in Argentina. The aims of the present study were: - to correlate FHB severity with F. graminearum DNA levels in spikes, - to determine the levels of DON in wheat grain and flour samples collected from the wheat growing region from Argentina, - to quantify F. graminearum both by a microbiological method and using TaqMan real-time PCR, and - to evaluate the quality of the grains. On wheat spikes obtained from the field, a good correlation was observed between disease severity and F. graminearum DNA levels. From 69 wheat grain samples evaluated, 56 (81%) showed DON contamination in levels ranging from 0.4 to 8.5 ppm, mean 2.4 ppm. From the contaminated wheat grain samples, 4 groups were selected based on the DON content. From each group, 3 flour samples were evaluated for DON contamination. Nine flour samples showed DON levels approximately 50% lower than the levels detected in grains. The microbiological examination of the wheat grain and flour samples showed F. graminearum contamination. Also most of the samples including grains and flour showed detection of F. graminearum genomic DNA using the TaqMan real-time PCR assay. The grain quality indicators such as weight per 1,000 kernels (g), test weight (Kg/hl) and protein content (%) were altered in comparison with a non-epidemic year.

**Mycotoxins and Fungal Drugs in Foods**

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Food-borne filamentous fungi produce a large number of extrolites, both in pure culture and on foods and feeds. Some of these are toxic to vertebrates (mycotoxins), others have bioactivities that may influence human and animal health in alternative ways. The most important mycotoxins are in general aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes, zearalenone, sterigmatocystin, 3-nitropropionic acid, cyclopiazonic acid,
penitrem A, verrucosidin and penicillic acid. However other extrolites may be important. Penicillin, produced by *Penicillium rubens*, *P. chrysogenum*, *P. nalgiovense* and *P. griseofulvum*, may be produced in foods and may make a minor contribution to penicillin resistance in bacteria. Mycophenolic acid, produced by the common food-borne fungi *P. brevicaespurnum*, *P. bialowiezense*, *P. roqueforti* and *P. carneum*, is a very efficient immune-system inhibitor, and thus pave the way for bacterial infections if accumulated in foods. Compactin, produced by *P. solitum* and *P. hirsutum*, and mevinolin, produced by *Aspergillus terreus*, are very effective cholesterol-lowering compounds. These may be seen as positive contributions to healthy foods, but they are not under medical control, as they would be if prescribed by a general practitioner. Fungal associations to foods and extrolite cocktails, the Gulliver effect and problems concerning drugs in foods will be discussed.

**FOOD COLORANTS FROM FUNGI**

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Most water-soluble natural food colorants origin from plant sources and thereby depends on a supply of raw materials. An alternative source is production of colorants by filamentous fungi in biotechnological-engineered cell factories. Naturally pigment producing filamentous fungi offers sustainable and non-genetically modified (non-GMO) sources of colorants with possibilities of obtaining high yields and quality control over the pigment profile. While production of fungal carotenoids as food colorants is well established, production of water-soluble polyketides is being investigated. Careful chemical profiling of fungal species have resulted in a palette of fungal strains producing many different colours and hues; however, not all are easy to use in a bioreactor setting for pilot scale production. The effect of extrinsic factors on metabolic regulation of growth and polyketide pigment production by chemotaxonomically selected potentially safe fungal strains of *Talaromyces* spp. known to produce *Monascus*-like polyketide pigments, was studied in a chemically defined medium. It was demonstrated that pigment production is affected quantitatively as well as qualitatively by the medium components, especially carbon and nitrogen sources. However, also the cultivation parameters in bioreactors had an impact on the yield. In conclusion, the results so far point towards potential future safe, wild type cell factories for the production of red and/or yellow pigments as natural food colorants.

**CHLOROGENIC ACID, A METABOLITE IDENTIFIED IN TOMATO FRUITS BY A METABOLOMICS APPROACH, IS INHIBITORY AGAINST THE BIOSYNTHESIS OF ALTERNARIOL BY ALTERNARIA ALTERNATA**

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Tomato fruits and processed tomato products are often reported to contain the mycotoxins alternariol (AOH), alternariol monomethyether (AME) or tenuazonic acid (TEN). All three mycotoxins are produced by the fungus *A. alternata*. Tomato products often contain moderate to low
concentrations but may occasionally contain very high amounts of these toxins. These results show that tomatoes are a typical habitat for \textit{A. alternata} and that the production of AOH, AME or TEN is highly dependent on the environmental conditions, e.g. the substrate composition of tomato varieties. In fact in a laboratory experiment a strain of \textit{A. alternata} showed a reduced production of AOH on a tomato model medium compared to other media. This result indicates that despite the fact that tomato is a common substrate for \textit{A. alternata}, it might contain AOH inhibiting substances. For this reason an untargeted metabolite profiling of various tomato varieties was performed by GC x GC/MS analysis. Tomato fruits of the same set of varieties were infected with \textit{A. alternata} in parallel. One variety proved to be more resistant against growth and AOH biosynthesis of \textit{A. alternata}. Metabolome data revealed that the concentration of chlorogenic acid was substantially higher in this variety. In subsequent growth experiments it could be demonstrated that purified chlorogenic acid indeed has a concentration dependent moderate growth inhibiting, but a strong AOH biosynthesis inhibiting effect. Transcriptional analysis of AOH biosynthesis genes by Real-Time PCR revealed a correlation between gene expression and AOH biosynthesis. These results indicate that chlorogenic acid might be one of the metabolites, which reduce alternariol biosynthesis in more resistant tomato varieties. Beside the reduction of AOH biosynthesis also a reduced colonization could be observed on this tomato genotype. This is in agreement with the recent observation that AOH is a pathogenicity or colonization factor. The presence of increasing AOH concentrations led to stronger colonization which seems to be counteracted by the presence of increased amounts of chlorogenic acid.

**Occurrence of the Alternaria Toxins Tentoxin, Dihydrotentoxin and Isotentoxin in Foods**

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Control of mycotoxins in foods is important, as some of them cause acute diseases and some may cause chronic symptoms through low-level intake over the long term. In the present study we investigated the \textit{Alternaria} toxins tentoxin (TEN), which is a phytotoxin and induces species-selective chlorosis$^{1,2}$, as well as the naturally occurring dihydrotentoxin (DHT) and isotentoxin (isoTEN). A stable isotope dilution LC−MS/MS method was developed for quantification of those toxins using chemically synthesized triply deuterated internal standards$^3$. Among the analyzed commercial food samples, 50% of juices, 80% of oils and 80% of seeds, respectively, were contaminated with TEN; in 33% of juices, 40% of oils and 80% of seeds, respectively, DHT was detected; whereas isoTEN was not quantifiable. The only exceptions containing isoTEN were apple chips samples processed via microwave vacuum drying. They were contaminated with an almost equal amount of isoTEN and TEN. This indicated an effect of the microwave vacuum process on TEN as an irradiation-sensitive substance. Juices from berries and cherries were all contaminated. The TEN concentrations ranged from 0.06 µg/kg to 0.18 µg/kg. The detectable DHT in juice was mostly not quantifiable. TEN was detected in all of 6 sunflower oil samples with the highest amounts (at an average of 4.83 µg/kg) found in organic first cold-pressed sunflower oil samples. In contrast to this, in rapeseed and thistle oil there was very little or no contamination. The highest contamination level in seeds was found in hemp seeds with 17.4 µg/kg of TEN and 11.8 µg/kg of DHT. The measured nut samples showed very little or no contamination with the toxins.
SESSION 4: INFLUENCE OF ENVIRONMENTAL FACTORS ON THE PHYSIOLOGY OF FOODBORNE FUNGI
CHAIRPERSON ROB SAMSON

FUNGAL SPORES AS VEHICLES FOR DISPERSION IN SPACE AND TIME

Jan Dijksterhuis

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The majority of fungal species form single- or multi-celled structures that play an important role in the worldwide distribution of fungi. Fungi belonging to the genera *Aspergillus*, *Cladosporium* and *Penicillium* are so well equipped in forming spores that they can be found in virtually every cubic meter of air that is sampled. Other fungal species as *Talaromyces macrosporus*, *Neosartorya spinosa* or *Byssochlamys spectabilis* form ascospores that are arguably the most stress-resistant eukaryotic cells described to date. In fungal spores biomolecules are stabilized and have become protected against stressors including high temperature, drought and reactive oxygen species. As a result of stabilization, living cells or organisms can survive extended periods of time in a virtually unchanged state. This contribution deals with aspects of dormancy and the breaking of dormancy in fungal spores. We follow the fate of spores during germination and will see that the change from dormancy to vegetative cell is impressive.

ENVIRONMENTAL STRESS AFFECTS FUNGAL COLONISATION AND METABOLIC PROFILES IN STORED WHEAT AND MAIZE

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Colonisation of wheat and maize by *Fusarium graminearum* and *Fusarium* section *Liseola* species occurs under conducive water activity (aw; 0.90-0.98) and temperature (20-30°C) conditions. This results in contamination with type B trichothecenes and zearalenone, and fumonisins respectively. This also results in concomitant dry matter losses which can result in mycotoxin contamination levels exceeding the EU legislative limits. Less information is available on the effect of such environmental stresses on other known mycotoxins which may also be present. We have used a targeted metabolomics approach to examine the effect of such interacting environmental stress on the range of known mycotoxins present and the relative amounts produced. This showed that 12-15 known mycotoxins were produced in wheat when stored at 0.98 aw and 25-30 °C. However, at 0.95 aw and 0.90 aw the number of mycotoxins produced decreased with quantities also decreased. For maize there were up to 14 known mycotoxins produced. Some were only produced under 0.95-0.98 aw and 25-30 °C. Interestingly, under environmental stress there was a switch from fumonisin B1 to B2, B3 and B4. The quantities and range of known mycotoxins produced was also influenced by storage conditions. These results are discussed in the context of the possible changes which may occur in mycotoxin profiles under climate change factors.
INTEGRATING ECOPHYSIOLOGY AND GENE EXPRESSION DATA TO UNDERSTAND Fusarium verticillioides PRODUCTION OF FUMONISINS

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A systems approach was used in order to predict the fumonisins accumulation by Fusarium verticillioides. Growth and phenotypic FB1 and FB2 production was studied in a range of environmental conditions including water activity (aw; 0.995-0.93) and temperature (20-35 °C). The effect of these conditions on the activation of the biosynthetic FUM genes was studies in parallel. Nine genes belonging to the fumonisins biosynthetic pathway gene cluster were selected (FUM1, FUM7, FUM10, FUM11, FUM12, FUM13, FUM14, FUM16, and FUM19) and their relative expression studied using microarray analysis. These datasets, including growth, fumonisin production and gene expression, were used to develop a mixed-growth-associated product formation model and link this to a linear combination of the expression data for the 9 genes. The model validation was carried out by examining data sets outside the model fitting conditions used (35 °C). This model is important in developing an integrated systems approach to develop preventions strategies to control fumonisin biosynthesis and could also be used to predict the potential impact that climate change factors may have on toxin production.


SALT (NaCl) CONCENTRATION AFFECTS GROWTH AND TEMPORAL OCHRATOXIN A PRODUCTION BY Penicillium nordicum STRAINS ON A HAM-BASED MEDIUM

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The ecological conditions found in dry-cured ham throughout the ripening process favours surface growth of a mycobiota population. Although in general mould colonization contributes positively to develop the desired characteristics typical of the final product, some species, especially Penicillium nordicum may contaminate the product with ochratoxin A (OTA). OTA is the most important mycotoxin found in dry-cured hams and other meat products. Because many cured meat products contain a high amount of NaCl they provide a specialised ecological niche for the OTA producing Penicillia. The objective of this study was to study the effect of two different NaCl concentrations (10 and 22% = 0.94 and 0.87 water activity (aw)) on growth and OTA production by three strains of P. nordicum on a ham-based medium. Media were inoculated with spore suspensions of the strains and incubated at 25°C for 12 days. Growth and OTA quantification was made every two days. This showed that there was faster growth at 0.87 aw than 0.94 aw on the ham-based media. However, there was no intra-strain difference in growth in these two conditions. Two of the three strains were OTA producers. Temporal production was optimum after 10-12 days depending on strain. One of the producer strains produced higher OTA at 0.94 aw than 0.87 aw. The other strain produced similar OTA amounts at both aw levels. The implications for these results are discussed.
**Mannitol is Essential for the Development of Stress-Resistant Ascospores in Neosartorya Fischeri**

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The group of extreme heat-resistant fungi, to which Neosartorya fischeri belongs, is a well-known group of food spoilers. Heat-resistant fungi produce extreme heat-resistant ascospores, which can survive temperatures as high as 95 °C for several minutes and are able to survive mild food preservation treatments such as pasteurization. The sugar alcohol mannitol is one of the main compatible solutes in N. fischeri and accumulates in conidia and ascospores. Biosynthesis of mannitol mainly depends on mannitol 1-phosphate dehydrogenase (MPD). In this study, a functional analysis was performed of the MPD encoding gene mpdA of N. fischeri. Expression of mpdA as judged by the fluorescence of dTomato under the control of the promoter of this gene was observed in aerial hyphae and conidiophores, but was highest in cleistothecia and ascospores. Disruption of mpdA reduced mannitol as much as 85 % of the wild type level. Trehalose levels had increased in the mutant to over 400 %, but specific oligosaccharides in the ascospores were absent. Decreased mannitol accumulation had no effect on mycelial growth with or without exposure to temperature- and oxidative stress. Some increase of the sensitivity of conidia against temperature- and oxidative stress was observed in the mutant. The most distinct phenotype of mpdA disruption was the complete absence of fully formed ascospores. Cleistothecia were formed in unaltered numbers, as were numerous asci, but cleistothecia collapsed after drying. Ascospore initials were formed as judged by electron microscopy and staining of nuclei. After 6 days of development many ascospores showed a distorted cell contents and malformed ascospore cell wall. The ascus cell wall however remained intact in mutant strains, while in the wild type the ascus cell wall disappeared and numerous ascospores accumulated in the ascomata. Addition of the MPD inhibitor nitrophenide to the wild-type strain resulted in disturbed ascospore formation. These results suggest a novel function for mannitol during fungal growth and development.

**Mutual Regulation of the Biosynthesis of Ochratoxin A and Citrinin in Penicillium Verrucosum as an Adaptation to Different Habitats**

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The FAO estimates that up to 25% of the world’s crop harvest is contaminated with filamentous fungi or their mycotoxins. For this reason it is important to understand the regulation of mycotoxin biosynthesis in order to develop prevention strategies to reduce the mycotoxin contamination of food and feed. In this study, the regulation of the biosynthesis of ochratoxin A (OTA) and citrinin (CIT), two hepato- and nephrotoxic mycotoxins produced by P. verrucosum, were investigated. In P. verrucosum the production of OTA and CIT is mutually regulated. On NaCl-rich media the biosynthesis of CIT is reduced, whereas that of OTA is increased. It could be shown that the production and excretion of the chloride-containing OTA molecule ensures cellular chloride-homeostasis under hypersalinic growth conditions.
Changes in the concentration of NaCl in the environment are transmitted to the transcriptional level by the HOG-MAPK signal cascade, which results in an adaptation of gene expression. Ochratoxin biosynthesis seems to be regulated by the Hog-MAPK-pathway. Western Blot experiments showed a correlation between the phosphorylation status of the HOG1-homologue of *P. verrucosum* and induction of OTA biosynthesis. Inactivation of *hog1* in *P. verrucosum* by gene knock out abolishes OTA biosynthesis under high NaCl conditions. In contrast, under oxidative stress conditions the biosynthesis of OTA is reduced and CIT production is increased instead. Changes in the oxidative status are often transmitted to the transcriptional level by a G-protein/cAMP/PKA signal cascade. In the current analysis a correlation between internal cAMP-levels and biosynthesis of CIT could be demonstrated. External application of cAMP resulted in a reduced biosynthesis, suggesting the involvement of such a signal cascade in the regulation of CIT biosynthesis. Citrinin is described to have antioxidative properties, which makes the induction of CIT biosynthesis under oxidative conditions favorable for the fungus. These results suggest that in *P. verrucosum*, the biosynthesis of OTA or CIT apparently act as an adaptation mechanism to hypersaline respectively oxidative stress conditions.

**Changes in the Physical state of the cytoplasm and Accumulation of compatible solutes are Related to the Stress Resistance of heat-resistant Ascospores**

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*Neosartorya fischeri* forms extreme stress-resistant ascospores that have a thick cell wall and are constitutively dormant. They are resistant to heat, freezing, drought, and high-pressure. Due to their ability to survive mild food preservation treatments, they are found in pasteurized food products. In this study we related heat resistance, the accumulation of compatible solutes and the physical state of the cytoplasm during ascospore maturation making use of Electron Spin Resonance (ESR) spectroscopy. Stress resistance was related to a highly viscous and anisotropic cytoplasm as was indicated by a high rotation correlation time of a spin probe. In addition, the redox status (chemical stability), as derived from the temperature dependence of the probe signal intensity, was determined. In early stages of ascospore maturation (11 to 15 days) ascospores accumulate compatible solutes (oligosaccharides and mannitol), which reduces the bulk water in spores. At later stages of maturation (15 to 50 days) no structural changes could be observed from the ESR spectra. However, the chemical (redox) stability still increased in the later stages of maturation (15 to 50 days). The change in the compatible solutes (less mannitol, more oligosaccharides) correlates with the increase of chemical stability and could increase the stability of the cytoplasm. Further we investigated spores in a dry state, because ascospores survive prolonged times in such state and drying increases the heat resistance. By using probes with different hydrophobicity we investigated the structure and chemical stability of different cell compartments. This technique has never been used on fungal spores before and although it is speculatively, it seems that the cytoplasm reacts different on drought stress than the more vulnerable lipid membrane. These results give novel information about the mechanism of heat-resistant ascospores and show that ESR spectroscopy is a useful technique to study heat-resistant ascospores.
**Comparison of survival of Cladosporium and Penicillium species during different developmental stages under low water activity**

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Fungi can grow under widespread environmental conditions. Many different fungi are found in varying food products and indoor environments. Indoor fungi are present in a considerable part of the European dwellings and cause cosmetic and structural damage. The presence of fungi poses a potential threat to human health as a result of continuous exposure as they are able to form allergens and mycotoxins. Fungal growth does not exist without the presence and availability of water. Not much is known on the response of fungi to humidity dynamics during different stages of their development. Relative humidity (RH) and water activity ($a_w$) are used in many studies for the amount of water available for the fungus. A RH of 80% ($0.8 a_w$) or higher is associated with fungal growth. On average the RH is below 50% in normal buildings. In order to study the fungal response to humidity dynamics, polycarbonate membranes containing two fungal species, Cladosporium halotolerans and Penicillium rubens, were placed in vessels with fixed relative humidity of 85%, 75%, 58% and 33% and transferred again to high humidity conditions after a week. The different developmental stages of C. halotolerans and P. rubens before and after periods of lower humidity are determined by using Cryo Scanning Electron Microscopy (CryoSEM). A different response to humidity dynamics was seen between several developmental stages and both fungi used. More in depth research will be done on the specific cellular response of the fungi to humidity dynamics.
SESSION 5 - OPEN SESSION

CHAIRPERSON LUDWIG NIessen

A REAL-TIME MULTIPLEX LOOP-MEDIATED ISOThermal AMPLIFICATION (LAMP) ASSAY FOR SIMULTANEOUS DETECTION OF IMPORTANT TRiCOTHECENE PRODUCING FusariaM spp. IN WHEAT

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Trichothecene mycotoxins such like deoxynivaleneol (DON), nivalenol (NIV) and T2-Toxin are produced by a variety of Fusarium spp. on cereals in the field and may be ingested by consumption of commodities and products made thereof. The toxins inhibit eukaryotic protein biosynthesis and may thus impair human and animal health. Aimed at rapid and sensitive detection of the most important trichothecene producing Fusarium spp. in a single analysis, a real-time multiplex loop-mediated isothermal amplification (LAMP) assay was set up. Two sets of LAMP primers were designed independently to amplify a partial sequence of the tri6 gene in Fusarium (F.) graminearum and of the tri5 gene in F. sporotrichioides, respectively. Each of the two sets detected a limited number of the established trichothecene producing Fusarium-species. However, combination of the two sets in one multiplex assay enabled detection of F. graminearum, F. culmorum, F. cerealis, F. sporotrichioides, F. langsethiae and F. poae in a group specific manor. No cross reactions were detected with 90 other fungal species or with cereal DNA. To demonstrate the usefulness of the assay, 100 wheat samples collected from all over the Bavarian state of Germany were analyzed for the trichothecene mycotoxin DON by HPLC** and for the presence of trichothecene producers by the new real-time multiplex LAMP in parallel analyses. LAMP reactions were positive in all samples with DON levels exceeding 40 ppb.

**Acknowledgement: data were provided by J. Rieder, Bayerische Landesanstalt für Landwirtschaft, Lange Point 6, 85354 Freising, Germany

DEVELOPMENT AND APPLICATION OF A LOOP-MEDIATED ISOThermal AMPlIFICATION ASSAY FOR RAPID DETECTION AND IDENTIFICATION OF AFlatoxigenic MOLDS IN FOOD SAMPLES

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Aflatoxins are carcinogenic secondary metabolites produced by several members of the genus Aspergillus in section Flavi with Aspergillus (A.) flavus, A. parasiticus, and A. nomius. We describe the development and evaluation of loop-mediated isothermal amplification (LAMP) assays for rapid detection of the three species in separate analyses. The acl1-gene of A. flavus and amyl1-genes of A. nomius and A. parasiticus, respectively, were used as target genes. The detection limits of assays were 2.4, 7.6 and 20 pg of pure DNA/reaction for A. flavus, A. nomius and A. parasiticus, respectively. For specificity testing, DNA extracted from mycelia of representative strains of 39 Aspergillus species, 23 Penicillium species, 75 Fusarium species and 37 other fungal species was used as template for the three LAMP primer sets developed. No unspecific reactions were observed. The LAMP assay was combined with a DNA extraction method for the analysis of pure fungal
cultures and artificially contaminated Brazil nuts, peanuts, green coffee beans as well as naturally contaminated Brazil nut samples. Assays were sensitive enough to detect $10^1$ and $10^2$ spores per reaction for *A. nomius* and *A. flavus*, respectively. Analysis of pure cultures of 68 isolates of *A. flavus* and *A. nomius* from Brazil nuts** showed that the LAMP assays had an accuracy of 83.8% when morphological identification was used as reference. Analysis of naturally infected Brazil nuts** showed that LAMP assays had a negative predictive value of 61.5% and 11.1% while the positive predictive value was 42.1% and 61.5% for *A. nomius* and *A. flavus*, respectively. Combining the results of both LAMP assays and comparing them with total aflatoxin contamination of samples revealed a positive predictive value of 77% for total aflatoxin contamination of samples.

**Acknowledgement:** cultures and Brazil nut samples kindly provided by M. Taniwaki, ITAL – Food Technology Institute, Av. Brasil, 2880, 13 070-178 Campinas SP, Brazil

**OCHRATOXIN A MIMOTOPES FROM AN EVOLVED PHAGE LIBRARY AND ITS APPLICATION IN IMMUNOASSAY**

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Ochratoxin A (OTA) is a mycotoxin produced by *Penicillium* and *Aspergillus*, and is believed to have toxic effects on human health. Mimotopes that mimic OTA obtained from phage display random peptide libraries have been used as substitutes for mycotoxin itself in immunoassays. We employed a phage display-based directed evolution to create mimotopes that bind OTA monoclonal antibody (OTA-MAb) with high selectivity. Sequence analysis of first-generation OTA mimotopes revealed a conserved FQ(N) LH sequence. Selected phage with this motif were found to bind OTA-MAb with IC$_{50}$ values of 0.50-1.03 ng/ml. In order to improve the selectivity for OTA-MAb, a secondary library in which the initially selected motif was conserved and flanking residues re-randomized was constructed. Post-selection mimotopes containing FQLH motif exhibited IC$_{50}$ values as low as 0.05 ng/ml, approximately 10-fold lower than that of first-generation mimotopes. A sensitive and selective enzyme-linked immunosorbent assay (ELISA) for OTA analysis was developed with OTA mimotopes instead of OTA. This demonstrates the potential of phage peptides for replacement of mycotoxins, and applications in immunoassay.

**GENOME AND TRANSCRIPTOME OF XEROMYCES BISPORUS, A MODEL (?) XEROPHILE**

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*Xeromyces bisporus* is a filamentous ascomycete which has the distinction of actively growing at the lowest water activity reported to date for any organism (0.62 $a_w$). It grows optimally at 0.89 $a_w$, a water activity which represents a significant osmotic stress for most other foodborne fungi. *X. bisporus* causes occasional spoilage of dried products, and typically has been isolated from sugary substrates such as dried fruits, liquorice and chocolate, although it has also been isolated from animal feed and tobacco. Moulds are the main cause of spoilage on foods where water availability is reduced, and their ability to grow in such conditions is key to their exploitation of this unique niche. We hope to uncover some of the molecular strategies employed during growth at reduced $a_w$ by comparing genome and transcriptome data with physiological
studies of *X. bisporus*. The genome of *X. bisporus* was assembled *de novo* and annotated by comparison with the *Aspergillus nidulans* genome. The transcriptomes of *X. bisporus* growing at optimal (0.89) and at extremely low water activity (0.68) were compared. Changes in the membrane fatty acid unsaturation index (UI), and in accumulation and leakage of the compatible solute, glycerol, over a range of water activities was also compared between *X. bisporus* and the xerotolerant *Aspergillus niger*. Sensing and adaptation to osmotic stress is regulated by the HOG (High Osmolarity Glycerol) pathway, which appears to be widely conserved among fungi, including the xerophilic foodborne basidiomycete *Wallemia sebi*. Elements of this pathway were indeed upregulated in *X. bisporus* under osmotic stress. Two genes involved in glycerol synthesis were upregulated at low $a_w$, and *X. bisporus* produced approx. twice as much glycerol per cm² colony as *A. niger*, much of it leaked into the agar. Both species increased glycerol production in conditions of osmotic stress, namely, low $a_w$ for *A. niger*, and both high and low $a_w$ for *X. bisporus*. The membrane fatty acid UI of *X. bisporus* was generally lower than that of *Penicillium* and *Aspergillus* spp., and showed only a gradual decrease with lowered $a_w$. No genes involved in fatty acid synthesis and desaturation were significantly differentially transcribed, in contrast to the halophile *Hortaea werneckii*. It appears that organisms employ different strategies to modify membrane fluidity during osmotic stress. Gene clusters encoding for secondary metabolites were not identified in *X. bisporus*, which supports the lack of metabolites identified by HPLC; this suggests that its ability to outcompete other moulds at very low $a_w$ is its main strategy for survival.

**Novel insights in the mode of action of polyene antibiotics**

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Formation of (carcinogenic) mycotoxins by food-spoiling fungi and the developing resistance of fungi against the preservatives benzoate and sorbate, are an ongoing concern for the food and feed industry. Macrolide polyene antibiotics were first discovered during the late forties and are effective against a wide range of fungal species. In the seventies and eighties, compelling evidence has been presented that this class of antibiotics targets ergosterol, the main sterol of fungal membranes. Subsequent research documented that different types of polyene antibiotics display different modes of action despite that they share a common target. Larger polyenes like amphotericin and nystatin can form complexes with ergosterol that become pores in the plasma membrane. This results in the collapse of vital ion gradients and loss of cell components resulting in death of the cells. The smaller uncharged filipin cannot form pores but act by a different mechanism. Together with sterols, filipin forms large complexes that are located between the leaflets of the lipid bilayer, resulting in disruption of the membrane. Natamycin like the other polyene-antibiotics specifically binds to ergosterol in the membrane, but we show evidence that its mechanism is different from the other compounds. Novel insights demonstrate that sterols may have different functions in the cell with respect to membrane trafficking, vesicle fusion and even activity of transporter proteins.
HEAT-RESISTANCE OF *Hami*G*er*a AND *Thermoascus* STRAINS ISOLATED FROM PASTEURIZED FRUIT-BASED FOODS

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Fruit-based beverages or semi finished products are usually subjected to a pasteurization process that can sometimes be ineffective to kill ascospores from heat-resistant moulds. With regard to this, although the most occurring and economically relevant heat-resistant species belong to *Byssochlamys*, *Neosartorya*, *Talaromyces*, and *Eupenicillium* genera, at SSICA an increasing number of rarely studied heat-resistant isolates have been recently detected as spoiling microorganisms in such products. The objective of this study was to establish thermal inactivation rates for two of those heat-resistant isolates, *Hamigera avellanea* and *Thermoascus crustaceus* in blueberry and grape juice and in a 12.5 °Bx buffered glucose solution, in order to assess their importance as spoilage fungi in pasteurized fruit-based foods. *Hamigera avellanea*. Of the two substrates, the blueberry and grape juice exhibited lower D values (19.23, 6.45 and 0.65 minutes, respectively at 87, 90 and 95 °C) than those calculated for the 12.5 °Bx glucose solution (23.81, 7.63 and 0.75 minutes, respectively at 87, 90 and 95 °C). The z-values calculated from the thermal death time curves did not prove to be affected by the heating medium, that being 5.39 °C in blueberry and grape juice and 5.27 °C in 12.5 °Bx buffered glucose solution. *Thermoascus crustaceus*. Of the two substrates, the blueberry and grape juice exhibited lower D values (35.71, 5.81 and 1.54 minutes, respectively at 90, 93 and 95 °C) than those calculated for the 12.5 °Bx glucose solution (55.56, 9.81 and 1.74 minutes, respectively at 90, 93 and 95 °C). The z-values calculated from the thermal death time curves did not prove to be affected by the heating medium, that being 3.69 °C in blueberry and grape juice and 3.37 °C in 12.5 °Bx buffered glucose solution. Since the heating times needed for the complete inactivation of *Hamigera* and *Thermoascus* ascospores proved to be comparable to or even longer than those applied to common heat-resistant species, the variation of pasteurization parameters does not seem to be a possible way to avoid product spoilage.

MYCOTOXIN BIOCONTROL: TWO EXAMPLES OF INTRA- AND INTER-SPECIFIC BIOCOMPETITION

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Aflatoxins (AF) and ochratoxin A (OTA) are two of the most abundant food-contaminating mycotoxins, produced by several *Aspergillus* and *Penicillium* species; the first is considered one of the most potent hepatocarcinogenic agents in man and animals, while the latter is reported to have carcinogenic, nephrotoxic, and immunosuppressive effects in long-term exposed individuals. The natural occurrence of these mycotoxins in foods represents considerable health risk to humans. As biocontrol agents, many species of bacteria, fungi and yeasts have been tested for their ability in reducing mycotoxigenic fungi and resultant mycotoxin contamination in food and
feed commodities. Regarding to biological control of aflatoxin occurring in oil-containing crops such as maize, peanut, and cottonseed, great successes have been achieved through the application of competitive atoxigenic strains of *A. flavus* and *A. parasiticus*. In meat derivatives such as salami and hams, various attempts to control the surface colonization by potential ochratoxinogenic moulds have been recently carried out: use of fungi as biocontrol agents against *P. nordicum* seems to be a particularly promising way to improve food safety without affecting properties and sensory quality of meat products. Here, two different approaches in setting and developing inter- and intra-specific biocompetition strategies to control mycotoxins in foods are discussed. A simple, high throughput fluorescence-based procedure, suitable for assessing the AF production in the medium during fungal growth, is used to screen a closed population of aspergilli from maize kernels sampled in the Lokobe Integral Reserve (Madagascar); an *A. oryzae* wild strain effective in reducing AF accumulation by aflatoxicogenic strains of *A. flavus* and *A. parasiticus* during biocompetition trials performed both in a synthetic medium and on a natural substrate (corn grains). On the other side, some atoxigenic strains of *P. nordicum* have been analyzed for their potential in preventing/lowering OTA accumulation during biocompetition with OTA producing *P. nordicum* strains in YES-liquid medium. The most effective strain identified by this procedure will be used for competition experiments on cured meat (salami).

**RECONSTRUCTION OF THE ORIGINAL FUNGAL FLORA OF HEAT-PROCESSED MEAT PRODUCTS BY PCR-SSCP**

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Food processing of spoiled meat is prohibited by law, since it is a deception and does not comply with food safety aspects. In general, spoilage of meat is mostly caused by bacteria. However, a high contamination level of fungi could be also found in some meat or meat products with certain preserving conditions. Moreover, spices used to flavor meat products were often contaminating with fungi. In case that unhygienic meat and raw material are used to produce heat processed products, the microorganisms will be deactivated by heat, so that they cannot be detected by a standard cultivation method. Therefore, this study aimed to apply a molecular biological method – polymerase chain reaction and single strand conformation polymorphism (PCR-SSCP) – to reconstruct the original fungal flora of heat processed meat. A new primer pair ITS1/ITS5.8R was developed and tested for its specificity for fungal but not plant and animal DNA. The level of detection of fungal DNA tested with thermocycling-PCR was down to 50 cfu/50 mg (corresponding with 10³ cfu/g meat) both in minced meat samples collected from supermarkets and spiked meat sample. This primer pair and PCR-SSCP method was finally applied with field samples: one soy sauce and 15 heat processed meat products which were already flavored with spices. 32 DNA bands in acrylamide gel were sequenced – all originating from fungal species, which were, in other studies, reported to contaminate meat e.g. *Alternaria alternata*, *Aureobasidium pullulans*, *Candida rugosa*, *C. tropicalis*, *C. zeylanoides*, *Eurotium amstelodami* and *Pichia membranifaciens*, and / or spices such as *Botrytis aclada*, *Guignardia mangiferae*, *Itersonilia perplexans*, *Lasiodiplodia theobromae*, *Lewia infectoria*, *Neofusicoccum parvum* and *Pleospora herbarum*. The result of this study confirms the applicability of
primer pair ITS1/ITS5.8R and PCR-SSCP method to specifically detect fungal DNA in heat processed meat products, and thus provides an overview of fungal species contaminating raw material such as meat and spices.

**Aspergillus section Flavi and aflatoxins in sugarcane juice and dried yeast**

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Brazil is the biggest producer of sugar cane and is responsible for 30% of world production, reaching 533 million tons in 2011-2012. This production is mainly due to the high demand of the ethanol and sugar industries. To obtain ethanol, yeasts are necessary, which are responsible for the fermentation of concentrated cane juice. In this process, the dried yeast is a by-product and is valued for its high protein content and as a source of proteins and amino acids for feed and food. There are studies that have shown the presence of Aspergillus flavus and A. parasiticus in sugarcane and surrounding soil and this occurrence can be related to the presence of aflatoxins in the dried yeast. However, there are few studies and more are necessary. The aim of this study was to investigate the source of aflatoxins in dried yeast. For this the presence of Aspergillus section Flavi was investigated in sugarcane and dried yeast as well as the aflatoxin contamination. Sugarcane juice (34), dried yeast (39) and soil (8) samples were collected from different production facilities in Brazil and were analyzed for aflatoxigenic fungi using dilution plating. Aflatoxin contamination was evaluated in sugarcane juice and dried yeast. The higher infection of Aspergillus section Flavi was found in the sugarcane juice with an average infection of 2.4 x 10³ CFU/mL, ranging from <10 to 3.0 x 10⁴ CFU/mL. The average infection in dried yeast and soil were <100 CFU/g and 400 CFU/g, respectively. From the total of Aspergillus section Flavi isolated, 91% were producers of aflatoxins B and G. The average of total aflatoxins obtained in dried yeast was 3.57 µg/Kg, varying from undetected to 10.19 µg/Kg, and the median was 2.50 µg/Kg. Most of the samples (74%) showed detectable levels of aflatoxins. In sugarcane juice the levels were lower, with an average of 0.55 µg/Kg, ranging from undetected to 4.09 µg/Kg. The median was lower than the detection limit (0.37 µg/Kg). Morphologically it was not possible to obtain the identity confirmation of the isolates. Through the phylogenetic tree constructed by β-tubulin gene sequencing, the species studied were distinguished from other Flavi groups. It is possible that the isolates are new species.

**Toxigenicity of Alternaria spp. isolated from grapes of Slovak origin**

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The aim of this study was to discover the ability of the Alternaria isolates, obtained from Slovak grape berries, to produce mycotoxins in vitro. A total of 44 analysed grape samples were taken from the various wine-growing regions of Slovakia in 2011 and 2012. Isolates were gained by direct placing of grape berries on DRBC plates (dichloran, rose bengal and chloramphenicol agar). After re-inoculation of the Alternaria genus representatives on PCA (potato-carrot agar) we identified 4 species-groups: A. alternata, A. arborescens, A. infectoria and A. tenuissima. A total of 188 randomly selected strains have been re-inoculated on YES (yeast extract agar) and tested for the ability
to produce mycotoxins alternariol (AOH), alternariol monomethylether (AME) and altenuene (ALT) by means of thin-layer chromatography. The results showed that the isolates of *A. infectoria* species-group (14 strains tested) are not able to produce the tested mycotoxins. On the other hand, isolates of *A. alternata* (44 strains tested), *A. arborescens* (39) and *A. tenuissima* (91) species-groups showed a high potential to produce these metabolites. Totally 56.8% of *A. alternata* species-group isolates produced ALT, 95.7% AME and 97.7% AOH. Further 64.1% of *A. arborescens* species-group isolates synthetized ALT, 82.0% AME and 94.9% AOH. Together 60.4% of *A. tenuissima* species-group isolates produced ALT, 75.8% AME and 73.6% AOH. Until today, these mycotoxins are not standardly monitored, but on the basis of the obtained results, it is clear that we should pay more attention to their occurrence in the grapes and grape products.

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**Fusarium toxins in Potatoes and Potato Products**

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Dry rot of potato caused by *Fusarium* spp. is a worldwide occurring disease which may cause the production of mycotoxins. Because the potato tuber is still metabolically active after harvesting, metabolized mycotoxins, e.g. glucosides, might also be detected. So far, only few studies were published concerning the incidence of *Fusarium* toxins in potatoes and potato based products from retail markets. Two HPLC-MS/MS methods for the matrix potato were established and validated, including a total of 19 *Fusarium* toxins (type A and B trichothecenes, zearalenone). The limits of quantification ranged between 0.2 µg/kg and 18 µg/kg. A total of 206 samples from retail markets were analysed. In 30.6 % of all samples levels of *Fusarium* toxins were detected. More than one single toxin (up to seven) could be found in 13.1 % of those samples. Type A trichothecenes such as DAS, 4-MAS, 15-MAS, SCRP, T-2, HT-2 and NEO were detected in raw potatoes, mashed potatoes, potato dumplings/dumpling dough, potato fritter and pre-boiled potatoes. Maximum concentrations from 9.7 µg/kg (NEO) – 253.9 µg/kg (15-MAS) were observed in raw potato tubers. Deoxynivalenol and zearalenone also occurred in numerous potato based products such as potato dumplings/dumpling dough, mashed potatoes, raw potatoes, potato fritter and chips/potato wedges. In fried potatoes and crisps no positive signal for one of the monitored mycotoxins could be observed. All mycotoxin positive samples were also analysed for glucoside metabolites. The identity of these conjugates was confirmed by mass spectra (product-ion-scans) and the incubation with glucosidases. In 3.4 % of the retail samples amounts of MAS-glucoside, SCRP-glucoside and HT-2 glucoside were detected. The conjugated toxins were found only in mashed and raw potatoes.

**Fungicides effectively used for growth inhibition of several fungi could induce mycotoxin biosynthesis in toxigenic species**

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Seven different commercial fungicides (Aliette, Rovral, Cantus, Ortiva, Luna Experience, Fenomenal and Mancozeb) were tested for their ability
to inhibit the growth of the fungal species *Penicillium nordicum*, *Penicillium verrucosum*, *Verticillium dahliae* and *Cladosporium spec*. In the case of the mycotoxigenic strains of *P. nordicum* and *P. verrucosum*, the biosynthesis of ochratoxin and citrinin were determined. Interestingly, only individual fungicides were able to inhibit the growth of the analysed fungi to some extent. In case of *P. verrucosum* the fungicide “Rovral”, an iprodion belonging to the substance class of imidazols, led indeed to a decrease in the growth rate but to a strong induction of mycotoxin biosynthesis as it has been described earlier also for the strobilurins. Consequently before using a given fungicide in order to enhance the shelf-life of crops, the applicability of this chemical compound has to be tested not only regarding its ability to inhibit fungal growth but also on the level of secondary metabolite biosynthesis.

**Water activity and temperature effects on mycotoxin production by Alternaria arborescens**

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*Alternaria arborescens* is the causal agent of tomato stem canker and it was also found in tomato fruits affected by “black mold”. This species has been reported to produce the host specific AAL toxins together with some of the main *Alternaria* sp. toxins that can be found as food contaminants, alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TeA). AAL toxins (TA, TB, TC, TD and TE) have chemical structures similar to the carcinogenic *Fusarium* sp. toxins fumonisins. The objective of this study was to determine the effect of water activity (aw, 0.995, 0.975, 0.950) and temperature (6, 15, 20, 25 and 30°C) on mycotoxin production on a synthetic tomato medium of an inoculum of 10⁵ spores of the reference strain *A. arborescens* EGS 39128. Mycotoxins determination was performed at 7, 14, 21, 30 and 40 days. Quantification of AAL TA toxin was made by HPLC with fluorimetric detection after derivatization with OPA. For AOH, AME and TeA determination the culture material was extracted with methanol and clarified with ammonium sulfate. The extracts of AOH, AME and TeA were analyzed by HPLC with UV photodiode array detector. AAL toxins TA and TeA were produced in higher levels than AOH and AME. The optimum AAL TA production (214 µg/g) occurred at 0.995 aw after 7 days of incubation at 15°C. A temperature of 15°C was the most favorable for AAL synthesis at all aw levels. The optimum conditions for TA accumulation (83.69 µg/g) were 0.975 aw and 30°C. These same environmental conditions were also the most favorable for AOH (0.63 µg/g, 40 days) and AME (0.45 µg/g, 14 days) accumulation. In general, high aw levels favored the mycotoxin biosynthesis. All toxins were detected at low levels at 6°C after the whole incubation period. A safe storage temperature for tomato fruits and high moisture tomato products (aw>0.95) in relation to *A. arborescens* toxins might be established below 6°C. The results obtained in the present work could be extrapolated to assess the health risk posed by tomato fruits and tomato products due to this pathogen and its toxins.

**A survey of genes for ochratoxin and fumonisin biosynthesis to select both mycotoxin non-producers strains of Aspergillus niger**

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Aspergillus niger and A. awamori are important microorganisms used for biotechnological purposes; however, they may produce two harmful mycotoxins known as ochratoxin A and fumonisin B2. Remarkably, not all A. niger and A. awamori isolates produce fumonisin and ochratoxin, and all new and unknown A. niger and A. awamori isolates must be carefully checked for mycotoxin production before their industrial use. Due to the relevance of this theme, we have designed a set of fluorescent PCR primer-pairs to amplify fragments of genes encoding essential proteins for ochratoxin A and fumonisin B2 biosynthesis by A. niger and A. awamori. A fluorescent primer-pair specific to detect A. niger/A. awamori was also designed. All the primer-pairs herein designed were used in a multiplex PCR system to survey the presence of the genes FAD-binding oxidoreductase (fad), polyketide synthase (pks) and R-oxoamine synthase (fum8) of A. niger/A. awamori strains from different origins. As expected, a fragment of 190 bp was found in all strains analyzed, successfully identifying A. niger and its cryptic species. Fragments of 540 bp corresponding to pks gene were detected in all OTA producing strains, but not in OTA nonproducing strains. The presence of fum8 gene was detected in all fumonisin producing strains but also in a small number of FB2 non producers. Although the presence of fum8 gene may not assure that a given strain is a FB2 producer, it is important to state that the absence of this essential gene for fumonisin biosynthesis can assure a FB2 non producing fumonisin phenotype. Concluding, the mPCR proposed in our study may be useful to indicate the biosafety of A. niger/A. awamori strains. The visualization of a single amplicon A. niger/A. awamori species-specific is a secure guarantee that the strain could be used in industries.

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**INFLUENCE OF VARYING TEMPERATURE AND WATER ACTIVITY ON FUNGAL GROWTH, PRODUCTION OF AFB1, AND EXPRESSION OF AFLATOxin BIOSYNTHESIS GENES IN ASPERGILLUS FLAVUS ON ALMOND MEDIUM**

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Almonds may be infected by Aspergillus flavus, the major responsible source of aflatoxin contamination, representing a serious food safety hazard and cause of economic losses due to the border rejection of contaminated products. Aflatoxins are produced both on the field and during storage under various environmental conditions. In this study we monitored the influence of varying temperature (20°C, 28°C, and 37°C) and water activity (0.99, 0.96, 0.93, and 0.90 a_w) on fungal growth, aflatoxin B₁ (AFB₁) production and gene expression in A. flavus grown on almond enriched medium. Analyses were performed on fungal mycelium collected at different time points along the incubation period. Four aflatoxin biosynthetic genes (aflR, aflS, aflD, and aflO) were monitored. The highest growth rate and the highest AFB₁ production were observed in A. flavus grown at 28°C and at 0.99 and 0.96 a_w. At 28°C, the further lowering of a_w caused a reduction of AFB₁ contamination. Both high (37°C) and low (20°C) temperatures caused a decrease of fungal growth and AFB₁ production, with the higher temperature affecting more than the lower temperature at 0.99 and 0.96 a_w. At 20°C the growth was completely inhibited when a_w was set at the lowest levels (0.93 and 0.90). However, with lowering of a_w the risk of AFB₁ contamination may increase at prolonged time of incubation. Regarding
the expression of aflatoxin biosynthesis genes, the evidence was that in general the activation trend of the two regulatory genes (aflR and aflS) and of the two structural genes (aflD and aflO) mirrored the AFB1 accumulation profile. In correspondence with onset of AFB1 synthesis the structural genes were highly upregulated compared to their basal level and in greater degree than regulatory ones. Generally, when conditions were unfavorable for aflatoxin production, the transcription levels decreased, above all those of structural genes. The regulatory genes were always expressed, regardless of aw and temperature values, also in absence of AFB1 production.

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DETERMINATION OF OCHRATOXIN A IN BREAKFAST CEREALS AND SNACKS FROM THE UNITED STATES BY HPLC-FLD

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A method of reversed phase high performance liquid chromatography (HPLC) with fluorescence detection (FLD) was optimized for determination of ochratoxin A (OTA) in breakfast cereals and snacks. In sample preparation, different extraction solvents and clean-up techniques were compared and we adopted acetonitrile/water (80:20, v/v) and an immunoaffinity column (IAC). The method provided recoveries of OTA from all sample matrices spiked at 0.5 ng/g level in the range of 95–100%. The limits of detection and limits of quantification for OTA were 0.032 and 0.10 ng/g for corn, wheat, and rice based samples; 0.038 and 0.12 ng/g for oat based samples, respectively. This method was applied to 114 samples of processed products containing corn, oat, wheat, and rice collected from five metropolitan areas in the United States (U.S.) A total of 57 samples (50%) were contaminated with OTA in the range of 0.12 and 7.43 ng/g. Among the OTA contaminated samples, 53% were labeled as organic and 47% were conventional with mean concentrations of 1.21 and 2.00 g/g, respectively. There were ten contaminated samples, all from oat based products, exceeding the maximum limits for OTA by European Commission Regulation (3 ng/g) in cereal based products.

PROTEOMIC ANALYSIS OF A MUTANT STRAIN OF ASPERGILLUS WESTERDIJKIAE FOR PRODUCTION OF OCHRATOXIN A

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An Aspergillus westerdijkiae mutant strain with a drastic reduction in production of ochratoxin A has an interrupted gene similar to the An09g05800 locus of Aspergillus niger annotated as encoding a putative PHD (Rum1) transcription factor. This gene has been described as involved in chromatin-mediated regulatory processes. The analysis of the genomic context of A. niger, showed that the gene rum1 is not linked to any gene clusters related with mycotoxin biosynthesis. Here, we have developed a differential proteomic analysis between the wild-type (ITAL142) and the mutant (ITAL142-T10) strain grown in medium conducive to OA production, with the purpose of identifying proteins that may be under regulation of the PHD transcription factor. Out of 23 proteins identified in ITAL142, those found most frequently were stress response proteins (36.4%) and the 26S proteasome regulatory subunit (9.1%). In filamentous
fungi, mycotoxin production and sporulation processes may share regulatory elements. Then, an analysis on the production and morphology of conidia was carried in both strains in MEA and YES media. No alteration was found regarding conidiation in MEA, but a greater number of conidia were observed in YES medium. This study is the first to show proteins alterations in ochratoxigenic fungi due to mutation in a regulatory gene.

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**GENETIC AND MYCOTOXIN DATA ANALYSES REVEAL THE CONTAMINATION OF BRAZIL NUTS BY ASPERGILLUS PSEUDONOMIUS**

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Aflatoxins are reported to be produced mainly by *Aspergillus* ssp. in section *Flavi*. The taxonomy of this section is continually changing. At the present time, *Aspergillus* section *Flavi* involves 23 species, which can be grouped into eight clades. The *A. nomius* clade, includes the species *A. nomius*, *A. bombycis* and *A. pseudonomius*. *A. nomius* was described as one of the main culprits for the presence of aflatoxins in Brazil nuts. *A. bombycis* is found in Brazil nuts, but at very low frequency. *A. pseudonomius* is a new species, described in 2011, as a producer of aflatoxin B1 (but not G-type aflatoxins). In this study, we examined 40 isolates, previously identified as *A. nomius*, collected from Brazil nuts at different stages of the production chain. Genotyping was conducted based on polymorphic loci generated by RAPD and AFLP markers. Considering 0.6 as the minimum threshold for designating genetic similarity, two groups (A and B) were formed by the UPGMA cluster analysis. All 40 isolates were assigned to two clusters when the computer software Structure was run. Nucleotide sequences data from β-tubulin and calmodulin genes were obtained and aligned to those from the type strains of *A. nomius*, *A. bombycis* and *A. pseudonomius* deposited in the NCBI database. For both genes, the isolates representing the A-group determined by AFLP and RAPD analyses grouped into one cluster that includes the *A. nomius* type strain (CBS 260.88). Isolates representing the B-group grouped into another cluster that includes the *A. pseudonomius* type strain (NRRL 3353). This analysis revealed that 15% of all isolates are *A. pseudonomius*, and not *A. nomius*, as previously recognized. Interestingly, an analysis of aflatoxins data has shown that all isolates herein analysed produced aflatoxins B and G. As a conclusion, we reported here for the first time the presence of *A. pseudonomius* in Brazil nuts and the ability of this species to produce aflatoxins B and G.

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**POTENTIAL PRODUCERS OF OCHRATOXIN A ON GRAPE S OF SLOVAK ORIGIN**

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The aim of study was to detect potential producers of ochratoxin A from grapes berries, surface sterilized berries (endogenous mycobiota), damaged berries and grape juice. We analyzed 20 samples of grapes, harvested in the
year 2011 from various wine-growing regions in Slovakia. For isolating the species we used the methods of direct plating berries, surface-sterilized berries (using 0.4% freshly pre-pared chlorine), and damaged berries on DRBC agar (Dichloran Rose Bengal Chloramphenicol). For the determination of fungal contamination of grape juice, we used plate-dilution method and DRBC agar. The cultivation in all modes inoculation was carried out at 25±1 °C, for 5 to 7 days. After incubation, Aspergillus isolates were inoculated on the identification media. Representatives of the genus Aspergillus were isolated from 13 samples of berries, 7 samples of surface-sterilized berries, 4 samples of damaged berries and 9 samples of grape juice. Overall, representatives of aspergilli were detected in 90% of samples (75 isolates). In this work our focus was aimed on the detection of potential producers of ochratoxin A, belonging to the genus Aspergillus. Isolates potentially producing ochratoxin A (Aspergillus section Nigri and Aspergillus westerdijkiae) after their identification were inoculated on YES medium (Yeast Extract Sucrose Agar) and after 14 days of incubation at 25±1 °C, in the dark, we tested their ability to produce ochratoxin A using thin layer chromatography. From the 16 isolates of potential producers of ochratoxin A neither one of the isolates of Aspergillus section Nigri (13 tested) did produce ochratoxin A. One isolate of Aspergillus westerdijkiae, isolated from surface-sterilized berries, produced ochratoxin A. The potential ability to produce ochratoxin A by the isolates was confirmed by HPLC analysis.

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Fungi producing fumonisin B₂ in Brazil nuts

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The Brazil nut (Bertholletia excelsa) is a plant with vast economic importance in the Amazon region and is an important product exported by Brazil. However, low levels of technology and inadequate management of raw material favor the infection by fungi that produce toxins. The knowledge of the distribution of toxigenic fungi in foods is important because it provides parameters to control and prevent the production of mycotoxins. Fumonisin B₂ (FB₂) is a mycotoxin produced by Fusarium species and Aspergillus section Nigri, and is a concern for human health. The aim of this study was to assess the toxigenic potential for fumonisin B₂ production by Aspergillus section Nigri strains isolated from Brazil nuts and checked for contamination of food by this mycotoxin. We analyzed 200 strains previously identified morphologically as Aspergillus section Nigri isolated from Brazil nut samples, preserved in silica gel at a temperature of 5 °C, from the fungal collection at the Microbiology Laboratory of the Institute of Food Technology (ITAL), Campinas-SP. To test the potential of fumonisin B₂ production, isolates were inoculated on Czapek Yeast Extract Agar Sucrose 20% (CY20S). The toxin was extracted with methanol and identified by high performance liquid chromatography (HPLC) using o-phthalaldehyde reagent (OPA) for derivatization reaction. A total of 100 Brazil nut samples from the States of Pará, Amazonas and São Paulo at different stages of the nut production chain were analyzed using an immunoaffinity column for extraction and cleanup and HPLC for detection and quantification of fumonisin B₂. From a total of 200 isolates of Aspergillus section Nigri, 36 (18%) produced FB₂.
Fungal spoilage in frozen chicken nuggets

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The development of restructured breaded chicken products allow a better use of meat cuttings generated by the poultry industry. These food products meet consumer needs because they are pre-cooked and frozen, being easy to prepare and presenting a longer shelf life. One problem that has been observed in the frozen food industry is the growth of moulds on the surface of these products during extended storage time. The observation of fungal colonies induces product rejection by consumers and can consequently affect the trademark, reflecting in economic losses to the companies involved. Despite the importance to the industry, there are very few studies dealing with fungal development in frozen foods. The objective of this study was to identify the species of filamentous fungi spoiling 7 samples of frozen breaded chicken visibly moldy obtained from an industry customer service. Aseptically 225 mL of 0.1% peptone water was added in 25 grams of sample and the mixture was homogenized in a stomacher during 1 min. Serial dilutions were prepared and inoculated on the surface of Petri plates containing agar Potato Dextrose and agar Dichloran Glycerol 18%, both supplemented with chloramphenicol. The plates were incubated at 25 °C for 7 days and 5 °C for 21 days with weekly observations. Samples with visible fungal deterioration presented counts between 10¹ and 10⁸ CFU/g. The predominant species in both media and incubation conditions were Penicillium decumbens, Penicillium aurantiogriseum, Eupenicillium spp. and Penicillium commune. These species are able to grow under refrigeration temperatures and some have been reported to grow at temperatures below 0 °C. About mycotoxin production, P. aurantiogriseum can synthesize penicillic acid, verrucosidin and nephrotoxic glyopeptides, while P. commune is a cyclopiazonic acid producer. Thus, besides the deteriorating aspect and the consequent economic loss, fungal growth in these products is a problem of food security due to the risk of consumer exposure to mycotoxins. Analysis of the production steps are being conducted to investigate the source of contaminants, as well as studies on the behavior of these species in breaded chicken products stored at low temperatures.

Mapping the heat resistance of fungi under conditions of pH and sugar concentration associated with beverages

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Heat treatments are a proven means of inactivating microorganisms (including moulds and yeasts (fungi)) in foodstuffs such as beverages. However, the number of ‘heat resistant’ species of fungi is reported to be increasing. Work described here investigates how the age of a fungus, and the
pH and sugar concentration of the beverage impact on the heat resistance of that fungus. ‘Heat resistant’ fungal cultures were secured from commercial beverage producers. Methods for producing and preparing the heat-resistant structures (principally ascospores) of these microorganisms were developed. The structures were allowed to mature and were then challenged with heat at different ages, enabling the D-values to be determined. They were also heated in media at differing pH and sugar concentrations. The maximum heat resistance for yeasts was derived when cultures were at least 3 weeks old (using 60 °C). For moulds, cultures at least 6 weeks old were used (derived using 85 °C). Heat resistances in a given beverage with manipulated pH and sugar concentrations showed that a high sugar concentration had a protective effect. Our results showed that, on occasion, some yeasts were more heat resistant under more acidic pHs, which was contrary to expectation. This information has enabled us to work with beverage manufacturers to minimise the risks associated with these microorganisms, such that sources of the fungi are identified and product spoilage is prevented.