



UNIVERSITÀ
CATTOLICA
del Sacro Cuore

**Scuola di Dottorato per il Sistema Agro-alimentare
Doctoral School on the Agro-Food System**

cycle XXVI

S.S.D: AGR/12; AGR/15; CHIM/01

**Characterisation of the mycoflora associated to dry cured
pork meat products with focus on *Penicillium nordicum*
and its biocontrol**

Coordinator: Ch.mo Prof. Antonio Albanese

Candidate: Giorgio Spadola

Matriculation n. : 3911387

Tutor: Prof. Paola Battilani

Academic Year 2012/2013

Alla mia mamma e al mio papà con infinita gratitudine...

Table of contents:	pag
1. Summary of the thesis	1
2. Foreword:	5
<i>i. Cured meat</i>	5
<i>ii. Mycotoxins in dried cured meat</i>	12
<i>iii. Toxigenic moulds</i>	28
<i>iv. Moulds identification</i>	41
<i>v. Ochratoxins</i>	50
<i>vi. Occurrence of ochratoxin A</i>	65
<i>vii. Ochratoxin A toxicity</i>	68
<i>viii. Related pathologic evidences</i>	69
<i>ix. Laws</i>	71
3. Aim of the thesis	121
4. The surface mycoflora on Italian salami	123
5. MALDI TOF MS method development for <i>Penicillium</i> species discrimination	147
6. MALDI TOF MS as a rapid screening tool for contaminants in the food industry.	177
7. Native yeasts as potential biocontrol agents in cured meat products	217
8. Conclusions of the thesis	247
9. Perspectives of the thesis	251
10. Acknowledgements	255

1.Summary

Penicillium nordicum is an important contaminant of cured meat products, representing 10% and 26% of the *Penicillium* spp. isolated, respectively, from the air or the products in a survey managed in Italy (Battilani et al., 2007). Several *P. nordicum* cured meat isolates proved to be important producers of ochratoxin A, OTA (Sansom and Frisvad, 2004; Pietri et al., 2006; Battilani et al., 2010). Currently, the appropriate setting of environmental conditions (temperature, relative humidity and air circulation), is the only accepted tool to prevent the uncontrolled growth of *P. nordicum* inside dry-curing plants through a carefully structured Hazard Analysis Critical Control Point (HACCP) plan (Asefa et al., 2011; Virgili et al., 2012). Even if the HACCP system has been successfully applied in the food industry, there are food safety hazards not carefully considered. This is especially true with regard to mycotoxigenic hazards associated with animal food products. The term “mycotoxigenic hazards” is used by Asefa et al. (2011) to describe pathogenic yeasts and toxic secondary metabolites of toxigenic moulds that contaminate food products and affect food safety. Most HACCP plans in food processing activities, such as the production of cheese and dry-cured meat products, considered mainly bacterial agents (Arvanitoyannis and Mavropoulos, 2000; Barbuti and Parolari, 2002), even if such food products get often contaminated with mycotoxigenic fungi and their metabolites (Spotti et al 1989; Spotti et al., 2001a; Battilani et al 2007). Therefore, it should be crucial to define a HACCP plan specifically focused on the mycotoxigenic hazards.

The identification, control and standardization of the surface mycoflora of cured meat products is mandatory to preserve the productions safety and the consumers health. This is the context of the effectiveness and reliability evaluation for the *Penicillium* spp. identification methods of interesting species for food production.

In this context, the research project of this PHD thesis tried to fill some gaps of knowledge with the attempt to limit the mycotoxigenic risk in the cured meat products chain.

The following topics were faced:

1. study of the composition and dynamic of fungal microflora present on the surface of cured meat products (salami) and the air of seasoning environments taking into account the influence of some process parameters (starter inoculum, curing temperature, stage of seasoning).
2. development of a MALDI TOF MS method for the identification of *Penicillium* at species level for future direct screening perspectives of the microflora present on cured meat products.
3. comparison and integration of different techniques, as morphological, molecular and mass spectral analysis, for the identification of *Penicillium* species in cured meat products.
4. evaluation of selected yeasts, isolated from dry-cured ham surface, to compete with *P. nordicum* and to inhibit OTA accumulation in the perspective of their use as surface starter biocontrol agents.

The activities carried out to perform all the points listed above and included in the PHD project were organized year for year as follows:

Regarding topic 1, the project focused on the study of fungal population related to salami with the quantification and identification of the fungal population following a morphological approach (Pitt, 1979).

Penicillium spp. were largely dominant in the fungal population detected. The highest fungal presence on salami was observed in half of the curing stage, at the peak of the presence of fungi in the air of seasoning rooms. The different Na/K ratio in the salt added to minced meat influenced the population of yeasts grown on the surface of the salami, while no effects were observed on the development of *Penicillium* spp.. There was no

clear role of starter inocula with different microorganisms on the microbial population, while higher storage temperatures increased *Penicillium* population.

Topics 2 and 3 focused on the development of a spectrometric method of analysis (MALDI TOF MS). The study was performed developing a protocol for the fast screening of *Penicillium* population aimed to discriminate different species based on the fungal protein fraction expressed. The approach, finalized to the identification of different biomarkers, concerned the analysis of the intra-cell soluble proteins fraction characteristic of each species. The method developed was able to obtain good spectra quality and reproducibility. *P. citrinum*, *P. roqueforti* and *P. nalgiovense* reference (type, REF) isolates showed an adequate clustering, based on MALDI results, to allow the definition of group names in the dendrogram.

The MALDI TOF MS protocol developed was used to screen a set of *Penicillium* strains isolated in different cured meat and cheese plant environments and products. The study highlighted the relevance of joining different approaches to optimize *Penicillium* species identification.

The MALDI approach was compared with the other approaches described in literature and in particular with morphological and molecular approaches (Samson and Frisvad, 2004). *Penicillium* spp. identification based on morphology was confirmed as very difficult as described in literature (Samson and Frisvad, 2004). The scientific community commonly do not accept only morphology as mean of identification, but on the other side molecular tools are still poor and MALDI TOF MS approach still need to be improved. Anyway MALDI TOF MS approach seems promising for future applications.

Comparing the MALDI TOF MS technique with the molecular approach looking the *beta tubulin*, *CO1* described in literature and obtained by public sequences was still not exhaustive. All the dendrograms obtained with different genes sequencing presented several gaps or doubts, different for each gene tested while some evidence were common each other and with the MALDI TOF MS clustering. Clustering together different genes was not possible being very few the strains sequenced for more than 1 gene available in public data bases. A better clustering and comparison will be possible when all the strains considered in this study will be sequenced for CO1 and β tubulin (ongoing activity).

Based on this study, MALDI is an interesting approach but it is not yet completely clear if it will be sufficient alone for *Penicillium* identification at species level; the study shows, as at the moment, that this discrimination is possible only for species not phylogenitically closely related (species from different Sections, Samson and Frisvad, 2004).

Studies related to topic 4 focused on the evaluation *in vitro*, on meat portions, the ability of selected yeasts isolated from dry-cured ham surface to compete with *P. nordicum* and to inhibit OTA accumulation in the perspective of their use as surface starter cultures.

Three yeast strains were selected. Meat portions (50 mm in diameter and 10 mm in height) were aseptically removed from pork muscle including *Biceps femoris* and *semimembranosus* and processed according to Battilani et al. (2010). The experimental design was carried out in duplicate. Dry cured pork meat portions were placed in Petri dishes, shared by their a_w , inoculated with one yeast and centrally spotted with *P. nordicum* suspension as described in the factorial design of the chapter 7 in the thesis.

D. hansenii showed the greatest antagonistic activity against *P. nordicum*, irrespective of investigated conditions in terms of water activity, incubation time and concentration of microbial population on ham surface. The outcome of this trial demonstrated the potential use of selected yeasts as biocontrol agents able to inhibit *P. nordicum* growth and OTA accumulation in the perspective of a safer processing of cured pork meat.