Toxigenic potential of fungal mycoflora isolated from dry cured meat products: preliminary study

TABUC C°°, BAILLY JD°°, BAILLY S°°°°, QUERIN A°°, GUERRE P°°

°° *UPSP mycotoxicologie, Ecole Nationale Vétérinaire, 23 chemin des capelles, 31076 Toulouse cedex*
°°°° *Myco 2B, BP4, 31170 Tournefeuille*

SUMMARY

Fungal development on the surface of dry cured meat products actively participates to acquisition and improvement of organoleptic qualities of these products. However, uncontrolled development of contaminant mycoflora may also lead to alteration of the aspect of products and synthesis of mycotoxins. This preliminary study was done to evaluate fungal mycoflora of French cured meat products and to investigate the toxigenic potential of isolated strains. Thirty seven isolates belonging to 12 *Penicillium* species were identified. Eighteen strains are reported in literature as toxin producers. They were tested for ochratoxin A, citrinin and cyclopiazonic acid production. Among them, one strain of *Penicillium cyclopium* was able to produce low level of ochratoxin A. Three strains of *Penicillium viridicatum* were found to produce cyclopiazonic acid at level as high as 12 mg/kg in vitro culture on YES medium. These results are in agreement with studies done in other countries and they show that uncontrolled fungal development has to be limited on cured meat products.

KEY-WORDS : *Penicillium*, fungal mycoflora, dry cured meat products, toxigenic potential, cyclopiazonic acid, ochratoxin A.

RÉSUMÉ

Potentiel toxinogène des souches fongiques isolées des produits carnés séchés et affinés : étude préliminaire.

Le développement de moisissures en surface des produits carnés séchés et affinés participe directement à l'acquisition et à l'amélioration des qualités organoleptiques de ce type de produits. Toutefois, un développement incontrôlé d'une flore fongique contaminante peut être à l'origine d'une altération de l'aspect de ces produits voire conduire à la production et à l'accumulation de mycotoxines. Cette étude préliminaire a pour but de déterminer la nature de la flore fongique observée à la surface de produits carnés commercialisés en France et de tester le potentiel toxinogène des souches isolées. Trente sept souches, appartenant à 12 espèces différentes de *Penicillium*, ont été identifiées. Dix huit de ces souches sont connues comme pouvant être capables de produire certaines mycotoxines. Nous avons donc testé leur capacité à produire de l'ochratoxine A, de la citrinine et de l'acide cyclopiazonique. Une de ces souches, appartenant à l'espèce *Penicillium cyclopium*, s'est révélée être productrice de faibles niveaux d'ochratoxine A. Trois autres isolats de *Penicillium viridicatum* ont produit de l'acide cyclopiazonique à des concentrations pouvant atteindre 12 mg/kg après culture sur du milieu YES. Ces résultats sont en accord avec ceux obtenus dans d'autres pays et ils montrent qu'il faut éviter tout développement fongique incontrôlé en surface des produits carnés affinés.

MOTS-CLÉS : flore fongique, *Penicillium*, produits carnés, potentiel toxinogène, acide cyclopiazonique, ochratoxine A.

Introduction

The thin layer of moulds recovering cheeses and cured meat products (a) has an antioxydative effect and holds the colour, (b) allows the surface not to become sticky or slimy, (c) participates to the development of a characteristic flavour, due to decomposition of lipids and proteins, and (e) finally, it gives the product its typical appearance [6, 18, 23]. Usually, the fungal species used in this kind of food making are selected strains (i.e: *Penicillium camemberti, Penicillium roqueforti*) [14]. They have been tested for toxigenic potential before any introduction in alimentary product [8, 12, 13]. Unfortunately, uncontrolled fungal development may also occur on these products. It may then lead to different detrimental effects: alterations of aspect, technological properties and nutritive value, development of mycosis and allergy agents, and production of mycotoxins [19]. These fungal contaminants usually belong to the genus *Penicillium* and/or *Aspergillus* [1, 5]. In a recent study, we showed that casual contamination of cheese by *Penicillium citrinum* could lead to production and accumulation of citrinin in the product [2]. There are only few studies on the fungal mycoflora of cured meat products. They mainly concern Spanish, Austrian or German productions [15, 16, 17, 24, 28]. No study was done to determine fungal mycoflora of dry cured meat products commercialised in France and to explore toxigenic potential of fungal strains isolated from processed foods.

The aim of this study was to investigate fungal mycoflora on a few number of commercial dry cured meat products and to test the toxigenic potential of *Penicillium* isolated strains in order to determine if there is any hazard for consumers and if this topic should be further developed.

Material and methods

Solvents and reagents

All solvents and reagents were purchased from VWR international (Fontenay sous bois, France) and were analytical grade. Mycotoxins standards (citrinin, cyclopiazonic acid, and ochratoxin A) were purchased from Sigma (Saint Quentin Fallavier, France). Citrinin was dissolved in chloroform, Ochratoxin A in 95° ethanol and Cyclopiazonic acid in methanol to obtain 1 mg/ml stock solutions that were stored at -20°C.

TLC plates were silica gel 60 plates (Merk, Nogent sur Marne, France). For citrinin quantification, plates were were first dipped in 10% oxalic acid solution for 30 seconds and then air dried overnight before use.

Meat samples

Five dry cured hams were purchased from French commercial market. They were produced in southwest of France. They were chosen randomly among slightly mouldy ones.

Five ripened dry sausage were also tested. They were covered by a thin layer of mould and corresponded to usual aspect of such products.

Fungal count and identification

For dry cured ham: surfaces were totally scrapped with a sterile lancet and sample was suspended into 5% tween 80 solution. After dispersion and homogenisation, 1 ml of decimal dilutions was plated on both malt agar medium (2% agar, 2% malt, 50 ppm chloramphenicol) and salted malt agar medium (malt agar medium + 6% NaCl). Fungal colonies were counted after 3, 5 and 7 days of culture at 25 and 32°C. Results were expressed as CFU/cm^2 of meat.

For dry sausages, 25 cm^2 of surface were dispersed in 5‰ tween 80 solution and decimal dilutions were prepared and plated on the same media. Results were expressed as CFU/cm^2.

The different strains were isolated from plates by several planting out on Czapeck and malt agar media. Identification of fungal species was done according to Raper and Fennel [21] and Raper and Thom [22] by macroscopic and microscopic examination of isolated strains at 3, 5 and 7 days of culture.

Toxigenic potential determination

Potentially toxigenic strains were tested for ochratoxin A, citrinin and cyclopiazonic acid production after 15 days of culture (23°C) on Yeast Extract Sucrose medium (2% yeast extract, 16% sucrose). For cyclopiazonic acid production, strains were also incubated on sterile rice for the same time and at the same temperature.

Mycotoxin quantification

Toxins were extracted from both culture media by mechanical agitation in appropriate solvent. After filtration, extraction and concentration, the toxins were separated by thin layer chromatography. Mycotoxins were then quantified by spectrofluorodensitometry using a Shimadzu CS930 fluorodensitometer (Shimadzu Corp., Kyoto, Japan). Quantification was done by comparison with known amounts of standard spotted on the same plate. Briefly:

- Ochratoxin A was extracted by acetonitrile-4% KCl_{aq} (9-1), separated by migration in toluene- ethyl acetate-formic acid (5:4:1, vol/vol/vol) and quantified by fluorodensitometry at 333 nm [3]. Limit of quantification: 20 ng/g in the culture medium; limit of detection: 10 ng/g.

- Cyclopiazonic acid was quantified as already described by Landsen [11]. Briefly, culture medium was extracted by methanol-chloroform (1:1, vol/vol). After filtration, extracts were partitioned against chloroform and filtered on phase separator filters. Development system used was ethyl-acetate/isopropanol-ammoniac (20:15:10). After a drying step, cyclopiazonic acid was revealed on plates by spraying a 10% paradimethylaminobenzaldehyde solution. Quantification was done at 600 nm. Limit of quantification: 200 ng/g in the culture medium, limit of detection: 80 ng/g

- Citrinin was extracted by acetonitrile- KCl (4% in water) (9-1) acidified by H_2SO_4 to pH 3. Development system used was toluene/ethyl-acetate/formic acid (6:3:1, vol). Citrinin was then quantified by fluorimetric detection at 330 nm [22]. Limit of quantification: 20 ng/g in the culture medium, limit of detection: 10 ng/g.

Results and discussion

Fungal mycoflora of dry cured meat products

Typical macroscopic aspect of cured hams and sausages used in this study are shown in figure 1.

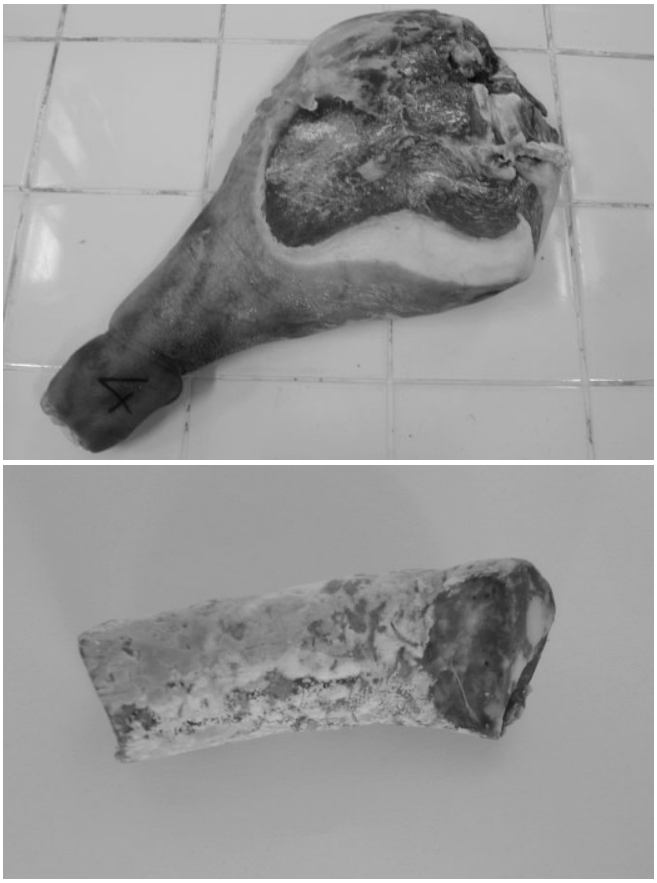

Figure I: typical macroscopic aspect of cured hams and sausages purchased from french commercial market.

Sample	Fungal genus	Malt agar	Salted malt agar
H1	*Penicillium*	183	91
	Aspergillus	0	366
	Yeasts	640	1282
H2	*Penicillium*	482	413
	Aspergillus	0	0
	Yeasts	2275	2000
H3	*Penicillium*	0,2	0,1
	Aspergillus	0	0
	Yeasts	179	344
H4	*Penicillium*	7,5	8,9
	Aspergillus	0	0
	Yeasts	136	0
H5	*Penicillium*	625	1312
	Aspergillus	0	0
	Yeasts	625	375
S1	*Penicillium*	600	350
	Aspergillus	0	0
	Yeasts	40	40
S2	*Penicillium*	250	290
	Aspergillus	0	0
	Yeasts	0	20
S3	*Penicillium*	300	120
	Aspergillus	0	0
	Yeasts	6800	7000
S4	*Penicillium*	520	350
	Aspergillus	0	0
	Yeasts	0	0
S5	*Penicillium*	500	110
	Aspergillus	0	0
	Yeasts	7000	7000

Table I: fungal numerations obtained for dry cured hams on malt agar and salted malt agar medium. Results are expressed in $x10^3$ CFU/cm^2. H: cured ham, S: ripened sausage

Results of fungal numerations are reported on table I. As estimated by macroscopic aspect, fungal mycoflora of cured hams was less developped than that of ripened sausages. Fungal isolates easily grown on salted malt agar medium. This result means that they are mainly xerophilic species. It is consistent with the substrates that are characterized by reduced water activity (<0,9) and high NaCl concentration (> 6%) [10]. The three cured hams presenting the higher contamination are those that have been ripened for the longest period. With one exception, all fungal isolates belong to *Penicillium* genus.

Thirty seven different strains belonging to 12 *Penicillium* species were identified (table II). *P. nalgiovensis* LAXA was the most represented one (30% of isolated strains) specially on sausages. This species, usually used as fungal starter in sausage processing, is not known to be toxigenic. Other non-toxigenic species such as *P. terrestre,* and *P. solitum* were isolated on hams only. Eighteen other isolated strains corresponding to *P. viridicatum* Westling, *P. palitans* Westling, P. *olivino-viride* Biourge, *P. cyclopium, P. expansum* Link, *P. crustosum* , *P. granulatum* and *P. steckii* are reported to be able to produce one or several mycotoxins such as patulin, ochratoxin A, citrinin or cyclopiazonic acid.

This mycoflora is consistent with those reported in the few articles describing *Penicillium* isolates to species level [17, 28]. These species are frequent contaminants of foods and feeds. Spores can be found in many environmental sources and conditions for ripening of dry-meat products (temperature, water activity and time) are very favourable to their growth. Contrary to several studies done on Spanish products, we isolated only one strain of *Aspergillus*. This isolate was identified as *Aspergillus fischeri* Wehmer which is not known to have any toxigenic potential. Such a difference between French and Spanish products could be explained by differences in the climate of the production areas. In France, cured meat products are usually manufactured in cold montain zones, unfavourable to Aspergilli development. Generally, French climate is not very favourable to the development of *Aspergillii* and it is now admitted that these species do not represent a real threat for human health in products that are completely processed in France [4].

Toxigenic potential determination

All potentially toxigenic isolates were tested for the production of cyclopiazonic acid, citrinin and ochratoxin A (table III). Patulin production was not tested since it has been demonstrated that this mycotoxin is not stable in meat products [12]. One strain of *P. cyclopium* isolated from ham was able to produce ochratoxin A at a low level. Three other *P. viridicatum* strains, isolated from three different samples of ripened sausages, were found to be able to produce cyclopiazonic acid at concentration as high as 12 mg/kg of culture medium. All these toxigenic strains were able to synthesise mycotoxins on YES medium and on autoclaved rice. Toxin levels were comparable in the two different media (data not shown). Although several studies already reported the toxigenic potential of fungal strains isolated from meat products, most of these studies focused on *Aspergillus* toxins such as aflatoxins and ochratoxins [9, 24, 25]. The production of

Fungal species	Serie	Possible toxins	Number of samples	
			S	H
P. viridicatum WESTLING	*viridicatum*	Cit., OTA, CA	4	0
P. palitans WESTLING	*viridicatum*	Cit, OTA, CA	0	1
P. olivino-viride BIOURGE	*viridicatum*	Cit, OTA, CA	0	3
P. cyclopium	*cyclopium*	OTA, CA	0	2
P. expansum LINK	*expansum*	Cit, Patulin	1	5
P. crustosum THOM	*expansum*	CA	0	1
P. granulatum BAINIER	*granulatum*	Patulin	0	5
P. terrestre JENSEN	*terrestre*		0	1
P. solitum WESTLING	*terrestre*		0	1
P. lanosum WESTLING	*commune*	CA	0	1
P, steckii ZALESKI	*citrinum*	Cit	0	1
P. nalgiovensis LAXA			7	4
A. fischeri WEHMER	*fumigatus*		1	

Table II: Fungal species isolated from cured ham and ripened sausages. Indicated toxins are those reported to be potentially produced by this fungal species. S: ripened sausage; H: dry cured ham. Cit: citrinin, OTA: ochratoxin A, CA: Cyclopiazonic acid

Strain (number of tested isolates)	Citrinin	Ochratoxin A	Cyclopiazonic acid
P. crustosum (1)	ND	ND	ND
P steckii (1)	ND	ND	ND
P lanosum (1)	ND	ND	ND
P cyclopium (2)	ND	ND, 260 µg/kg	ND
P expansum (5)	ND	ND	ND
P viridicatum (4)	ND	ND	ND, 3, 10, and 12 mg/kg

Table III: toxigenic potential of Penicillium isolates. Toxins were quantified after a 15 days culture period in optimal conditions for toxinogenesis. ND: not detectable.

ochratoxin A by *Aspergillus ochraceus* strains isolated from dry-cured meat products has been reported [9]. In our study, none of the *P. viridicatum* strains were able to produce Ochratoxin A even if this fungal species is known to be an important producer of this toxin in other substrates such as cereals [20]. By contrast, one strain of *P. cyclopium,* isolated from cured ham produced low level of Ochratoxin A. Taking into account the numerous (known and unknown) toxins in the *Penicillium genus,* some authors estimated the toxicity of extracts using biological tests [17, 28]. However, one recent study on toxigenic potential of *Penicillium* isolates found on Spanish fermented sausages reported production of cyclopiazonic acid by some of the *Penicillium* strains [15]. Citrinin production has also been reported, but none of our strains was able to produce this toxin [27].

Conclusion

On the whole, present results show that French processed cured meat products may be contaminated by potentially toxigenic *Penicillium* strains. The species observed in samples belong to species known to be frequent contaminants of meat products or correspond to fungal starters used in manufacturing. Among isolates, 4 of them were found to be able to produce cyclopiazonic acid at relatively high level. Although this result has to be confirmed by more extensive studies on a large number of samples, it suggests that uncontrolled fungal development should be limited to avoid mycotoxin contamination of dry cured meat products.

Aknowledgements

The authors would like to thank Marie-Rose Trumel for technical assistance and Joseph Le Bars for expert assistance and helpful discussion in the study of fungal contamination of meat products.

References

1.- ANDERSEN S.J.: Compositional changes in surface mycoflora during ripening of naturally fermented sausages. *J. Food Protect.,* 1995, **58**, 426-429.

2.- BAILLY J.D., QUERIN A., LE BARS-BAILLY S., BENARD G., et GUERRE P.: Citrinin production and stability in cheese. *J. Food Protect.,* 2002, **65**, 1317-1321.

3.- BOUDRA H., LE BARS P., et LE BARS J.: Thermostability of ochratoxin A in wheat under two moisture conditions. *Appl. Environ. Microbiol.,* 1995, **61**, 1156-1158.

4.- CASTEGNARO M., ET PFOHL-LEZKOWICZ A. Les aflatoxines. *In* Les mycotoxines dans l'alimentation: evaluation et gestion du risque. CSHPF, Tec et Doc., 1999, 199-248.

5.- DELESPAUL G., GUEGUEN M., et LENOIR J.: La flore fongique superficielle des fromages de Saint Nectaire et de Tomme de Savoie, son Èvolution au cours de l'affinage. *Rev. Lait. Fr.*, 1973, **313**, 715-729.

6.- DESMAZEAUD M.J., GRIPON J.C., LE BARS D., BERGERE J.L.: Etude du role des micro-organismes et des enzymes au cours de la maturation des fromages. *Lait,* 1976, **56**, 379-396.

7.- EL-KADY I., EL-MARAGHY S., et ZOHRI A.N.: Mycotoxin producing potential of some isolates of *Aspergillus flavus* and *Eurotium* groups from meat products. *Microbiol.* Res., 1994, **149**, 297-307.

8.- ENGEL G., et TEUBER M.: Toxic metabolites from fungal cheese starter cultures (Penicillium camemberti and P. roqueforti). *In* Mycotoxins in dairy products, VAN EGMOND (H.P.)., Elsevier Appl. Sci., London and N.Y., 1989, 163-192.

9.- ESCHER F.E., KOEHLER P.E., ET AYRES J.C.: Production of ochratoxins A and B on country cured ham. *Applied microbiol.*, 1973, **26**, 27-30.

10.- HERNANDEZ E;, et HUERTA T.: evolucion de los parametros

microbiologicos del jamon curado. *Microbiologia*, 1993, **9**, 10-19.
11.- LANDSEN J.A.: Determination of cyclopiazonic acid in peanuts and corn by thin layer chromatography. *J. Assoc. Off. Anal. Chem.*, 1986, **69**, 964-966.
12.- LE BARS J., et LE BARS P.: Strategy for safe use of fungi and fungal derivatives in food processing. *Rev. Med. Vet.*, 1998, **149**, 493-500.
13.- LE BARS J.: Cyclopiazonic acid production by Penicillium camemberti Thom and natural occurrence of this mycotoxin in cheese. *Appl. Environ. Microbiol.*, 1979, **38**, 1052-1055.
14.- LEISTNER L.: Mould-fermented foods: recent developments. *Food Biotechnol.*, 1990, **4**, 433-441.
15.- LOPEZ-DIAZ T.M., SANTOS J.A., GARCIA-LOPEZ M.L., et OTERO A.: Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of *Penicillium isolates. Int. J. Food Microbiol*., 2001, **68**, 69-74.
16.- MIZAKOVA A., PIPOVA M., et TUREK P.: The occurrence of moulds in fermented raw meat products. *Czech J. Food Sci*, 2002, **20**, 89-94.
17.- NUNEZ F., RODRIGUEZ M.M., BERMUDEZ M.E., et ASENSIO M.A.: Composition and toxigenic potential of the mould population on dry cured Iberian ham. *Food Microbiol.*, 1996, **32**, 185-187.
18.- PHILIPP S., et PEDERSEN P.D.: Mould cultures for the food industry. *Dan. Dairy Food Ind.*, 1988, **6**, 10-12.
19.- PITT J.I., et HOCKING A.D.: Fungi and food spoilage, 1985, Academic press, New York.
20.- POHLAND A.E., NESHEIM S., et FRIEDMAN L.: Ochratoxin A: a review. *Pure Appl. Chem.,* 1992, **64**, 1029-1046.
21.- RAPER K.B., et FENNEL D.I.: *The genus Aspergillus*. The Williams & Wilkins Compagny. Baltimore. 1965.
22.- RAPER K.B., et THOM C.: A manual of the *Penicillia.* Hafner Publishing Compagny. New York and London. 1968.
23.- RODRIGUEZ M., NUNEZ F., CORDOBA J.J., BERMUDEZ M.E., et ASENSIO M.A.: evaluation of proteolytic activity of micro-organisms isolated from dry cured ham. *J Appl. Microbiol.*, 1998, **85**, 905-912.
24.- ROJAS F.J., JODRAL M., GOSALVEZ F., et POZO R.: Mycoflora and toxigenic *Aspergillus flavus* in Spanish dry-cured ham. *Int. J. Food Microbiol*., 1991, **13**, 249-256.
25.- STRZELECKI E., LILLARD H.S., et AYRES J.C.: Country cured ham as a possible source of aflatoxin. *Applied Microbiol.*, 1969, **18**, 938-939.
26.- TRAN T.S., BAILLY J.D., QUERIN A., LE BARS P., et GUERRE P.: Fungal contamination of rice from south Vietnam, mycotoxinogenesis of selected strains and residues in rice. *Rev. Med. Vet*., 2001, **152**, 555-560.
27.- WU M.T., AYRES J.C., et KOEHLER P.E.: Production of citrinin by *Penicillium viridicatum* on country-cured ham. *Applied Microbiol*., 1974, **27**, 427-428.
28.- WU M.T., AYRES J.C., et KOEHLER P.E.: Toxigenic *Aspergilli* and *Penicillia* isolated from aged cured meats. *Applied Microbiol*., 1974, **28**, 1094-1096.