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Insights into existing and future fungal and mycotoxin contamination of cured meats

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Abstract

Cured meat products are widely consumed in various regions of the world and, as for other foods, consumers are increasingly aware of the need for better quality and safety. Various fungal communities can colonise meat products during their curing phase with beneficial or detrimental effects depending on the dominant colonisers. Some contribute to flavour, anti-oxidative and protective effects, while others cause spoilage, including undesirable off-flavours/odours and mycotoxins contamination. Recently, increasing research on fungal ecophysiology and mycotoxin occurrence, especially of ochratoxin A (OTA) and aflatoxins, in these products, has been shown. This review addresses the existing and new knowledge which is available to provide insights into the reasons why certain fungi colonise cured meat products including their ecology, the importance of critical control points and effective use of new monitoring methods for evaluating the risk of mycotoxin contamination of these products.

Introduction

Meat is especially rich in proteins, vitamins, and minerals and is an important element in the human diet [1]. Because of its perishable nature, meat historically has been conserved using different methods. One of the key strategies has been to preserve meats by curing. This has involved a lowering of the water content with salt, reducing the pH during the fermentation process, and stabilising the product by use of a variety of curing phases. This preserves and extends the shelf-life of meat products, which have become an important group of foods produced and consumed in Europe and exported worldwide [2,3]. There is a wide range of such cured meat products depending on the type of product, processing conditions and the region or country of origin [4,5]. The most internationally recognised are dry-cured hams and dry-fermented sausages mainly originating in Italy and the Iberian Peninsula such as Parma and Iberian dry-cured hams, and salami and salchichón dry-fermented sausages. Care is needed in describing the processing of cured meats because of the numerous types of cured meats which exist around the world. For example, in the case of hams, e.g., Mediterranean hams are characterised by a long drying period and eaten without further smoking or cooking, while those produced in northern European regions usually have a short ripening period and are smoked [4]. Focusing on the ripening process of Mediterranean hams, this consists of three fundamental stages, that may last for up to 36 months, from 16 to 25°C: salting, resting (post-salting) and drying/ageing [4,6]. In the case of dry-fermented sausages, the production consists of first comminuting and blending of the meat and fat (at 4°C), then, after the addition of spices, carbohydrates, salt (NaCl) and bacteria or yeast-based starter cultures, casings are filled under vacuum and the sausages are ripened/dried, favouring surface colonisation from the native mycoflora or as a result of inoculation with a mould starter culture [7,8,9], and temperature and relative humidity parameters are adjusted to

promote the moulds' growth (12-22°C). However, the nature of the mould colonisation on the surface of meat products has always been a matter for debate [12]. Yeasts are generally considered as favourable, either within or on the surface of the meat matrix or the casing [13]. In contrast, the presence of some filamentous moulds on the casing is considered desirable in Mediterranean style sausages, while they are unwanted in Northern types of sausages [15]. Like any other food fermentation, the microorganisms originated from the environment [16] and/or from contaminated products, such as salts [17] and spices, may then become adapted to occupation of the specialised niches developing in the cured meat matrix itself [20]. Most of these play a critical role in the development of the characteristic flavour and texture development of these cured meat products. Often these inoculants contribute to proteolysis and lipolysis phenomena during the ripening period [4]. However, some moulds may produce undesirable effects on the quality of these products resulting in off-flavours, black spots, floccose mycelium and characteristic colouration due to sporulation and conidial pigmentation on the surface of these products [23,24]. In contrast, others may affect the safety of cured meats by synthesising mycotoxins which are undesirable chemical contaminants with adverse effects on animal and human health [3]. In the case of colonisation of meat products by filamentous moulds the most challenging aspect is represented by the meat surface or by the casing. Here, the high lipid content and salt concentration and the low water activity (a_w), favour the growth of xerotolerant and xerophilic fungi [26]; mainly belonging to the *Penicillium* and *Aspergillus* genera. In general the beneficial species are *P. nalgiovense*, *P. chrysogenum*, *P. solitum*, and the recently identified species *P. salamii* [27], while *P. nordicum, P. verrucosum*, *A. westerdijkiae* and *A. ochraceus* are responsible for mycotoxin contamination and spoilage effects (off-odours and flavours) together with the black spots caused by *Cladosporium* species (*C. oxysporum*, *C. cladosporioides* and *C. herbarum*) [23,24]. In general, the presence of mycotoxins in cured meat products could be the result of either direct contamination with moulds [28,29] or carry over from contaminated ingredients used including the meat of animals being exposed to naturally contaminated feed, spices, pepper and salt [30,31,32,34]. The presence of toxigenic and spoilage filamentous mould species have often been reported in many kinds of meat products of different origin, from dry fermented sausages [35] to dry cured ham [36], culatello [37], to pinnekjøtt [38], from Norway [26] to Slovenia [39] and Argentina [40]. Results from studies in various countries have shown that OTA is the most important and frequent mycotoxin found contaminating dry-cured ham and dry-fermented sausages [7,29,32,41]. This mycotoxin is undesirable in foods because of its nephrotoxic, hepatotoxic and immunotoxic properties, with the kidney and liver the main targets of this toxin. It has been rated as a Group 2B carcinogen by the International Agency for Research of Cancer [43]. In addition, other mycotoxins besides OTA have also been found in some cured meats such as the carcinogenic aflatoxins (Group 1) or citrinin and, cyclopiazonic acid (CPA). Although the presence of mycotoxins in cured meats is a real risk for consumers' health, only a few reports have made an effort to make decisions on adjustment of the interacting conditions of temperature and a_w in relation to the processing of these products to be effectively utilised as part of a hygienic management system based on HACCP (Hazard Analysis and Critical Control Points). Many of the current research studies have been aimed at the impact of environmental conditions used for processing of high quality cured meat product processing in relation to the development of fungal colonisation and toxin contamination by spoilage moulds. In the following sections, new evidence from the literature has been taken into account, including the fungal ecology of toxigenic moulds on the key cured meat products, to provide an overview of the critical control points to monitor the mycotoxin risk and to provide strategies aimed at avoiding the contamination by mycotoxigenic fungi.

Fungal ecology aspects in cured meat production

To date, a significant amount of research has been carried out to relate the key environmental factors governing the maturation of cured meats, like temperature and a_w, with the environmental conditions that cause the production of toxic metabolites by the principal toxigenic species, predominantly responsible for mycotoxin contamination of cured meats. The strains of *P. nordicum* appear to have evolved to have

significant tolerance to the relatively high concentrations of salt used in the curing process [2,6,44]. It has been observed that such species is relatively tolerant of high concentrations of NaCl (15%=0.85 a_w), even in cured meat-based media [2]. Interestingly, on a dry-cured sausage-based medium modified with different NaCl amounts, *P. verrucosum* grew much faster than *P. nordicum* at 15-25^oC at 0.90 and 0.94 a_w. However, examination of the optimum and marginal boundary conditions for growth in relation to a_w x temperature interactions showed that the optimum growth was at 0.94 and 20°C for *P. nordicum* and 0.94-0.95 a_w and 25^oC for *P. verrucosum* [2,6,45]. In addition, there were significant differences in the a_w x temperature range for OTA production on this simulated cured meat-based medium, *P. nordicum* produced OTA at 0.85 a_w with an optimum at 15°C and 0.90 a_w. In contrast, optimum production by *P. verrucosum* occurred at 0.90 a_w over the whole temperature range (10-30°C) and was optimum under freely available water conditions (0.995 a_w). This suggests that *P. verrucosum* is much more sensitive to concentrations of NaCl used for curing meats, and OTA production was drastically reduced in the presence of KCl [46], while *P. nordicum* has the capability for growth and more importantly produces OTA under relatively stressed abiotic interacting conditions [47]. In addition, it is also important, due to recent findings, to consider the ecology of *A. westerdijkiae,* which can also contaminate meat with OTA. This species is capable of producing high concentrations of OTA, over a higher temperature range, than *P. nordicum* [48], although *P. nordicum* has a wider temperature range for OTA production with respect to *P. verrucosum* [45,49] and a shorter lag phase prior to growth [2]. This again suggests that *P. nordicum* is particularly adapted to dry-cured meat ecological niche [50]. Differences were also found amongst strains of the same species. In general, *A. westerdijkiae* has been found to be well adapted to the curing condition of ham (20-25°C). Where lower temperatures are necessary for curing of dry fermented sausages (16-12°C), these cooler conditions are more suitable for OTA production by *P. nordicum.* The adaptation of this species and *A. westerdijkiae* to NaCl-rich niches may be related to its ability to rapidly synthesise compatible solutes which allow its endogenous enzyme systems to operate under such extreme osmotic stress where many other contaminants cannot grow. Indeed, it has been suggested that OTA production by *P. nordicum* may be a key factor in the maintenance of chloride homeostasis [44,53]. In relation to aflatoxins and CPA in meat, data is very scarce, although *A. flavus*, *A. parasiticus* and *P. griseofulvum* have been frequently isolated from cured meat. However, AFB_1 and CPA were rarely found present. The synthesis of toxic secondary metabolites occurs only under some restrictive conditions and production does not necessarily correlate with growth [2], but these species have been proven to produce toxic secondary metabolites during ripening under conducive environmental conditions. For studies on the ecology of *A. flavus*, shorter lag phases than *A. parasiticus* was found, except at 15°C. However, for both species, the growth rates were quite similar, with optimum growth at 25°C and 0.95 a_w, with no growth at 10°C. For AFB_1 production, temperature and a_w levels necessary were ≥15 °C and ≥0.90 a_w, respectively. However, *A. flavus* produced much higher concentrations of AFB_1 than *A. parasiticus* [54]. Recently, the ecology of *P. griseofulvum* in the cured meat environment [55] has been studied, because CPA was found in meat products [56,57]. *P. griseofulvum* was found to be responsible for maximum CPA production in a dry-cured ham-based medium at 25 °C and 0.95 a_w. However, when inoculated in dry-fermented sausages, the production of CPA was important at cooler temperatures, even 12 °C, and increased during seasoning. This could be due to the intense meat proteolysis that releases free amino acids, such as tryptophan, a direct precursor of CPA [55]. Knowledge on the ecophysiology of these *Penicillium* and *Aspergillus* species on cured meat products could help to make appropriate technological modifications during the curing process for sausages and ham. Information on the optimum and marginal conditions for growth and mycotoxin production by toxigenic fungi are practically useful. The information can be implemented as part of a quality assurance (QA) system, to identify the possible risk of mycotoxigenic fungi colonisation and toxin contamination, driving the curing processes to be modified to try and minimise the risk of mycotoxin accumulation.

Diagnostics approaches for monitoring toxigenic fungi and mycotoxin risks in cured meats

Since the toxigenic fungi colonise the surface of aged products can lead to the accumulation of considerable amounts of toxins, especially OTA [29,41], it is important to have rapid and robust methods for monitoring the early presence of mycotoxigenic fungi and/or mycotoxins. In this respect, the early relative expression of mycotoxin related biosynthetic genes and phenotypic mycotoxin accumulation in cured meats during ripening has been correlated, and therefore represents an important critical control point that can be relatively easily monitored [60]. Also, the co-occurrence at different rates of beneficial starter cultures such as *P. nalgiovense* and harmful species like *P. nordicum* could be easily monitored using the LAMP assay [7]. By using RT-qPCR, the temporal changes in the expression of the *otapks* and *otanps* genes by *P. nordicum* and *P. verrucosum* in relation to OTA production on slices of dry-fermented sausage salchichón and dry-cured ham demonstrated that the gene expression was higher in *P. nordicum* than in *P. verrucosum* in both meat matrices. In particular, the *otapks* gene was overexpressed by both *Penicillium* species, especially in salchichón, and this could be explained by the different composition and a_w of these two matrices. The a_w values of dry-cured fermented salchichón were higher than those observed in ham (0.87 a_w), which could also partially explain the correlation between OTA production and the higher *otapks* expression, regardless of the ochratoxigenic species present [49]. Similar findings were found in relation to the expression of genes involved in aflatoxin production, in meat model systems. For *A. flavus*, a decrease of a_w, regardless of temperature, caused an increase in the expression of the regulatory *aflR* and *aflS* genes, and the *aflP* gene expression was higher when temperature and a_w were lower. For *A. parasiticus*, the highest and lowest expression values of the regulatory *aflR* and *aflS* genes were found at 0.95 a_w and 0.85 a_w, respectively. The expression of the structural *aflP* gene of both species was stimulated at low temperature and a_w levels. AFB_1 production showed a better correlation with the expression of the regulatory *aflR* and *aflS* genes in *A. flavus* than in *A. parasiticus* [61]. These recent studies demonstrate the usefulness of monitoring gene expression to evaluate the risks of mycotoxin accumulation in cured meats, and also are fundamental to identify the temporal critical control points during the curing phase of these products. In this respect, recently, some interesting publications have addressed this problem by developing molecular/chemical tools (LAMP assay, RT-qPCR, electronic nose) in order to rapidly identify the risks of toxin accumulation in cured meat, as early as possible [3,7,49,61,62].

Biocompetition between fungal strains for mycotoxins risk reduction, new evidence

Growth of toxigenic strains on the surface of dry fermented products such as dry-fermented sausages and ham could no longer be considered problematic if selected fungal starter cultures are used. Surface inoculation helps to minimise competition with the toxigenic moulds and ensures curing can effectively proceed [63]. Starter cultures thus could help to shorten the ripening period, ensure colour development, enhance flavour and improve product safety [6,65,66,67,68] counteracting the possible development of potential toxigenic moulds, and minimising the potential hazard related to OTA, and other mycotoxin contamination of these products. Colonization of vegetable products by toxigenic moulds could be controlled by effective antifungal methods, including the use of both chemical and physical treatments [69]. However, in cured meat products such approaches may be detrimental as they also inhibit beneficial moulds, which contribute to develop the unique sensory characteristics of cured meat products [73]. Conversely, such methods could even be responsible for the promotion of mycotoxin production since these toxic secondary metabolites are often synthesised in response to stress factors [74,75]. Biocompetition, by the application of protective cultures, could be considered the most valuable strategy to minimise the growth of common mycotoxigenic species and mycotoxin accumulation in dry-cured meat products [63,76,77,78]. The presence of yeasts and filamentous fungi during the curing phase is considered fundamental and beneficial for the improvement of the characteristic flavour and odours of the product. This is despite the potential risk of harmful microorganisms becoming established, especially when unwanted environmental contaminants colonize the surface. Common practices suggest that, removal of

the mouldy surface of dry-cured meats often seems sufficient to protect consumer health from mycotoxin contamination. However, this may not be enough as mycotoxins can diffuse inwards into the product, especially in sausages and less so in cured ham [79]. Thus, the application of a protective culture on the surface of dry cured meats has been widely investigated [80]. Yeasts, filamentous moulds, bacteria, together with other strategies, have been examined to try and reduce the presence of toxigenic or spoilage species on dry cured meats. This protective, competitive and also enzymatic action of these mycoflora has been very useful in preventing the diffusion of OTA, CPA and AFB_1 into the inner layers of cured meat products [79,81]. Amongst filamentous moulds, *P. nalgiovense* is the oldest fungal starter culture for meat ripening. It has been evaluated for its competitiveness against different toxigenic moulds [63], including *P. nordicum* at different rates [7], reducing OTA contamination on dry fermented sausages. *P. chrysogenum* has been shown to inhibit CPA production by *P. griseofulvum* in sausages [83]. PgAFP-producing *P. chrysogenum* RP42C was also demonstrated to preserve ham from OTA occurrence, for up to 9 months, with respect to other mixtures of selected autochthonous non-toxigenic moulds [8]. An almost atoxigenic *P. nordicum* strain [84], was also shown to reduce OTA accumulation by 90% on salami when co-inoculated with a toxigenic *P. nordicum* strain. However, it should also be noted that commercial meat starter cultures of bacterial origin and autochthonous yeasts exert varying responses, sometimes stimulating growth and OTA production by *A. westerdijkiae* and *P. nordicum* in different meat-based media [85]. Yeast strains, require the appropriate environmental conditions to exert their competitive antifungal ability, and need to be applied during the post-salting stage [86]. Interestingly, *D. hansenii*, one of the most promising yeast starter cultures for meat seasoning [87], has been shown to significantly reduce OTA production by *P. verrucosum* in a meat-based medium at 0.92 a_w, on dry-fermented sausages and on dry-cured ham slices. This was supported by data showing a decrease in the expression of *otanpsPN* gene [88]. *D. hansenii* has also been shown to stimulate AFB_1 production by *A. parasiticus* at 0.99 a_w, and conversely a reduction of *aflR* and *aflS* gene expression and toxin at 0.92 a_w in the meat-model system, in dry-cured ham and dry-fermented sausage slices [86]. The combination of PgAFP with *D. hansenii* provided a successful inhibitory effect on *A. parasiticus* growth as well as on aflatoxin production on sliced dry-fermented sausages [89]. *Saccharomycopsis fibuligera* and *D. hansenii* were tested against *Aspergillus ochraceus* and *P. nordicum* during speck production [90] and OTA was not detected. These are only experimental strategies proposed to reduce growth, production and contamination caused by toxigenic fungi. However, this type of information could help in driving producers to use the right coloniser or controller/biocompetitor as a basis for the development of different typical cured meat products.

Ochratoxin A and Aflatoxins in cured meat: is there a real risk?

In general, as discussed above, a heterogeneous indigenous fungal population growth on the surface of dry-cured meat products could lead to a potential risk for mycotoxin contamination and mainly, due to the ecological features of these products, to these toxic compounds accumulation. In fact, on such highly specialised foods, very few toxigenic moulds are able to grow and produce high concentrations of mycotoxins, partially favoured by the osmotic stress which they are exposed to on the surface of cured meat product during ripening. For example, dry-cured ham, sausages, bacon and other dry-meat products from Croatia and Bosnia Herzegovina, were contaminated by OTA and AFB_1 [96,97] and some with citrinin [98]. There are still few studies that monitor the presence of OTA and AFB_1 in cured meats. In this section, new information respect to the recent publications on mycotoxins risk in cured meats is summarised by last six years of surveys (Table 1). In particular, taking into account the only available mycotoxin recommended level for meat products (OTA <1 µg/Kg) set by Italian law [99], there is at least one product per survey that exceeds the limit, even up to one hundred times. In general, regarding the 1109 cured meat products listed in Table 1, about 13.8% were contaminated with OTA above the established limit, meanwhile, of the 558 samples analysed for aflatoxins content, around 4% resulted contaminated above a putative limit of 1 µg/Kg. Otherwise, OTA contamination above the limit equally occurs in sausages and hams, (14% and 12.9%, respectively). Due to the few data available on aflatoxins occurrence in cured meat, it is not possible to draw a scenario, but it seems that contamination of cured meat with this mycotoxin should be

considered as a possible threat and thus additional surveys are needs to evaluate the potential risk to consumers from this carcinogenic toxin. In addition, no data on CPA and citrinin are listed in Table 1 because there is still very few evidence of this kind of contamination. The first survey about the CPA contamination evidenced that 27 of 61 Iberian dry-cured ham samples were contaminated with CPA, with values ranging from 36.1 to 540.1 μg/Kg [57]. However, the ongoing consultation requests made to EFSA by the European Commission, will result in an opinion being probably provided in July 2019. The harmful effects of OTA levels discussed for other foods have not been considered for meat and derived products (Food Standards Agency - October 2018 Stakeholder update on rapidly developing policy on food contaminants; URL: https://www.food.gov.uk/news-alerts/consultations/october-2018-stakeholder-update-on-rapidly-developing-policy-on-food-contaminants). To date, only Italy has set a guideline value of 1 μg/Kg OTA in meat and derived products [99]. In our opinion, OTA could represent a real risk in cured meat products especially in artisanal uncontrolled production. In-depth studies focused on potential exposure risk to the groups of consumers which have a high weekly intake of such products need to be considered in combination with biomarker/toxicology studies to evaluate the possible need for implementation of EU regulation on this mycotoxin on meat. Based on the very limited data available on aflatoxins and CPA contamination of cured meats the potential risks to consumer health is low at the present time.

Conclusions

Cured meat production depends on various ecological factors (meat composition, variable temperatures and a_W) but also on the entire ecosystem of the diverse microbial population consisting of yeasts, bacteria and filamentous moulds. This review has shown that there are complex interactions between both the abiotic and biotic factors and fundamental knowledge of this ecosystem is critical to control and minimize fungal spoilage moulds and mycotoxins in these widely consumed products. This review shows that numerous ecological and molecular studies have been recently done to improve our knowledge of why some moulds are able to colonise these specialized niches and to better identify the principal risk factors in relation to mycotoxin accumulation in cured meats. Molecular tools like LAMP and RT-qPCR may provide very useful approaches for the early detection of risks of OTA contamination of cured meat products. However, there is a need for their development into more portable devices which can practically be utilized and implemented in this industry. Beneficial and competitive effects of different starter cultures have also been recently reported. This may provide promising strains of yeasts and filamentous fungi which are biocompetitive and can minimize the potential for mycotoxin contamination. However, it is important that they do not affect the typical sensorial and technological characteristics of the product. Finally, despite clear evidence of the real risk of OTA contamination of such cured meat products, Governmental food safety agencies largely not deemed this to be a priority and thus Europe-wide regulations for maximum mycotoxin thresholds in these products have not yet been set.

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In this work, the influence of water activity (aw), temperature and substrate composition on fungal growth and OTA production by *Penicillium nordicum* and *Penicillium verrucosum,* evidenced that both produce a large amount of OTA on dry-cured ham and "salchichón" slices in environmental conditions usually found during processing of dry-cured meat products.

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