Chapter

Fungal Contaminants and Mycotoxins in Nuts

Giulia Mirabile, Patrizia Bella, Antonio Vella, Vincenzo Ferrantelli and Livio Torta

Abstract

Contamination by fungi and mycotoxins in nuts has achieved much attention in recent years. In fact, the fungal metabolites produced by the species of *Aspergillus*, *Penicillium* (aflatoxins and ochratoxins), *Fusarium* (trichothecenes, zearalenones and fumonisins) and *Alternaria* (alternariotoxins) with toxic and/or carcinogenic effects are considered a threat to human and animal health. In this chapter we will discuss the main fungal *taxa* and related mycotoxins most frequently associated with these materials. In this regard, the first results on the level of contamination by fungi and mycotoxins in samples of almonds and pistachios of different origins will be reported. The main strategies to reduce the risk of contamination will also be recommended.

Keywords: nuts, contaminating fungi, mycotoxins, *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*

1. Introduction

Due to high nutritional value, pleasant taste and importance also in the pharmaceutical and cosmetic fields, nuts have been consumed by humans for thousands of years all over the world [1, 2]. Furthermore, some nuts are included in feed for several animal species. However, both the allergic forms to these products [3] and the health risks for prolonged and massive ingestion of mycotoxins present in these foods [4–6] are widely documented. Nut allergy is a hypersensitivity to some organic substances of this food caused by the consumer's immune system, whereas the risk of mycotoxicosis is related to the health of the nut. In particular, contaminating mycotoxins are produced by various saprophytic or phytopathogenic fungal microorganisms that can infect nuts and other foodstuffs throughout the whole production chain, from the farm to the plate [7].

Mycotoxins are a heterogeneous group of organic substances, generally characterized by low molecular weight, active at extremely low concentration (parts per billion, ppb, or parts per trillion, ppt; μg/L and ng/L, respectively), very resistant to degradation, produced by the secondary metabolism of numerous species of filamentous fungi (molds). When these microorganisms infect and develop on food and feed, they release the toxic molecules which, once ingested by the consumer (man or animal), can cause various acute (rash, vomiting, diarrhea, headache, etc.) or chronic (nephropathies, immunosuppression, carcinogenic effects, etc.) damages [8].

The health risks may arise from their carcinogenicity, so much so that the International Agency for Research on Cancer (IARC), based on epidemiological data, studies on cancer in experimental animals and mechanistic studies, has evaluated the carcinogenic risk of some mycotoxins in humans [9]. In order to protect the consumer from excessive exposure to the most dangerous mycotoxins, several Countries and federations of States have issued regulations that limit the maximum permissible content in a wide variety of foods for human and zootechnical use (for the EU, Commission Regulation (EC) No 1881/2006, Official Journal of the European Union, L. 364/5, 20.12.2006; Commission Regulation (EC) No 401/2006, Official Journal of the European Union, L70/12, 9.3.2006 and subsequent updates).

From the first report of a turkey disease ("turkey X disease") [10] associated with feeding these poultry with mycotoxin-contaminated peanuts (groundnuts), a large number of publications concerning the presence of fungi and their mycotoxins contaminating nuts have been spread all over the world. It is now known that among the mycotoxins most harmful to the consumer, listed by the IARC, are indicated aflatoxins, produced by fungi of the genus *Aspergillus*, ochratoxins produced by fungi of the genus *Aspergillus* and *Penicillium* and numerous others produced by different species of *Fusarium*, all capable of infecting nuts [7, 11]. Furthermore, several other mycotoxins, the "emerging mycotoxins" (alternariotoxins, enniatins, sterigmatocystein, etc.), produced by fungal species belonging to the aforementioned or to others genera (*Alternaria*), found in different nut samples, are currently being studied for their presumed dangerousness [12, 13].

2. Mycotoxins and associated risk to human health

The acute and chronic damages from massive and prolonged ingestion, inhalation or cutaneous absorption of mycotoxins are well documented and reported from all over the world.

From a historical point of view, the best known and described mycotoxicosis is ergotism (ergot of rye), widespread in some European regions since the Middle Ages. The disease is due to the ingestion of *Claviceps purpurea* sclerotia (rich in clavine alkaloids, lysergic acids, simple lysergic acid amides and peptide alkaloids), ground up with grains of various infected plants of cultivated and spontaneous species of the *Pooideae* subfamily (rye, millet, barley, oats, triticale, wheat, etc.). Two types of ergotism have been documented: convulsive and gangrenous. The symptoms included convulsions, hallucinations, skin burning and gangrene due to the constriction of the blood vessels, loss of hands and feets [14].

Several clinical studies have highlighted the correlation of mycotoxin effects with various pathological states in consumers, even if in most cases a "cause–effect" relationship is not clearly demonstrated [15]. Only for aflatoxins (in particular aflatoxin B1, AFB1) produced by some *Aspergillus* and *Penicillium* species, have been ascertained carcinogenic, hepatotoxic, teratogenic, and mutagenic effects on human health [16]. For this reason, IARC included the aflatoxins in the Group 1B: "Carcinogenic for humans" [17]. The risk of liver cirrhosis and immune effects, acute diseases such as hemorrhagic liver necrosis, edema and lethargy, up to death, have also been reported in frail individuals, children and in high-risk areas in developing countries [18–20]. Moreover, AFB1, once ingested by animals, is metabolized in aflatoxin M1 (AFM1) and excreted in the milk. Hence AFM1, included by IARC in the Group 2B, "Possibly carcinogenic to humans" [17], can be found in milk or milk products.

As regards ochratoxins, and in particular, ocrhatoxin A (OTA), carcinogenicity on laboratory animals is known, as well as damage to the kidneys, heart and liver injuries in humans. To date, there is no evidence of direct effects on the

appearance of carcinogenic phenomena on humans [21]. According to the IARC, OTA is included in the Group 2B [17]. OTA has been also associated with the Balkan endemic nephrophaty (BEN), disease affecting the rural populations of the Balkans and resulting in a high incidence of chronic kidney problems and cancers of the organs of the excretory system [22].

With reference to mycotoxins produced by fungi belonging to the genus *Fusarium*, the toxicological data indicate that fumonisins, trichothecenes (deoxynivalenol, T2 toxin, HT2 toxin) and zearalenone caused evident and known effects on humans, while others are considered a potential risk for consumer health. In particular, the fumonisins produced mainly by *F. verticillioides* and related species are suspected to cause esophageal cancer [23–26]. Fumonisin B1 is included in the IARC Group 2B [17].

Among the trichothecenes, deoxynivalenol (DON, known also as vomitoxin) produced by *F. graminearum* and related species, shows acute toxicity in animals (gastroenteritis, immunotoxicity, cardiotoxicity, refusal of feed, etc.) and poisoning in humans, with various symptoms such as nausea, vomiting, diarrhea and fever, as reported in China in the second half of the last century [27]. Because of the lack of data on its carcinogenicity in humans and only limited evidence on its carcinogenicity in experimental animals, DON is not classified by IARC [17]. T2 and HT2 (metabolized from the T-2 toxin after ingestion) toxins are considered agents of cytotoxic and immunosuppressive effects, which can cause acute intoxication and chronic diseases in both humans and animals. The fungal species most involved in the production of these secondary metabolites are *F. langsethiae*, *F. poae* and *F. sporotrichioides* [28]. The toxins derived from *F. sporotrichioides* are classified by IARC into Group 3 of carcinogenic substances [17]. Trichothecenes have been identified as the toxic agent in cases of Alimentary Toxic Aleukia (ATA, septic angina) associated with the consumption of moldy grain by both animals and humans in the USA, Japan, the former Soviet Union and elsewhere. The symptoms of the disease are characterized initially by general toxic stage (headache, weakness, fever, nausea, vomiting, gastroenteritis, etc.), followed by leukopenic stage manifested by changes in blood and, finally the angina-hemorrhagic stage [14].

Zearalenone (ZEA), mainly produced by *F. graminearum* and related species, does not cause acute poisoning in humans, but no studies on carcinogenic effects have been reported. ZEA caused an increased incidence of tumors in liver and pituitary cells in mice, but no carcinogenic effect was seen in rats, therefore considering limited carcinogenic effects in animals [17].

Aflatoxin, trichothecenes, ochratoxin A, fumonisins, zearalenone, fusarochromanone, have been shown to cause also immunosuppression and increase the susceptibility of animals to infectious disease [29].

Mycotoxins can pose several risks to human and animal health. The risks associated with health have often been characterized, however the mechanisms by which these toxins cause such damages have not yet well defined. Anyhow, the quantity and duration of exposure to mycotoxins, the synergy between the various fungal metabolites, the genetic predisposition and physical conditions of the consumer and other factors, can ultimately play a fundamental role in the manifestation of their toxic activity.

3. Main mycotoxigenic fungal genera in nuts

3.1 The genus Aspergillus

Aspergillus, a widespread Ascomycota genus belonging to the *Aspergillaceae* family, is divided in 6 subgenera and 27 sections with more than 400 species [30]. It

received its name from Michieli in 1729 that, viewing the microscopic structure of its conidiophore, was reminded of the device called in Latin "aspergillum", used to sprinkle holy water during the liturgy [31]. *Aspergillus* species grow most as saprophytes on decaying vegetable organic matter, but they can also colonize human tissues, home and hospital environments. Furthermore, *Aspergillus* is one of the most important genera with high economic and social impact due to its ability to produce mycotoxins, dangerous secondary metabolites with potential carcinogenic activity against humans and animals [32]. On the other hand, some species are employed in several industrial and food production processes (production of citric acid, gluconic acid, kojic acid, amylases, cellulases, hemicellulases, soy sauce and miso) [33].

In this fungal *Taxon*, conidiophore is the most important microscopic structure for its identification. During mycelial growth, hyphae called "foot cells" form a single conidiophore perpendicular to cell axis. The conidiophore presents a large apex that form a rounded or elliptical vesicle (columella). In uniseriate species (*A. fumigatus*), the fertile area of the vesicle is surrounded by a layer of cell called phialides that produce long chains of conidia or conidiospores (**Figure 1a**). In biseriate *Aspergillus* species (*A. niger*) another layer of hyphae called metulae exist between the vesicle and the phialides. In some *Aspergillus* species (*A. flavus*), conidiophores can be uni- or biseriate. The vesicle with phialides, metulae if are present, and conidiospores form the "conidial heads" [32]. The size of conidiophore and conidial head, the presence of metulae, the shape and the color of conidiospore are important identifying features. All these structures are typical of asexual reproduction, but in *Aspergillus* sexual reproduction also occurs and its microscopic features (cleistothecia, asci, ascospores, Hülle-cells) are also important for identification. Macroscopically, colonies are granular or with a suede-like surface consisting of dens felt of conidiophores. Growth is usually very fast and mature colonies are white, yellow, yellow-brown, dark brown-black, green, gray or light blue in color (**Figure 2a** and **b**) [34].

Aspergillus species are thermophilic, preferring hot humid climates but can grow in a wide range of temperature (7–42°C). Spores are usually present in aerosol and can be easily dispersed by air for long distances. When they find optimal conditions of temperature and humidity, they germinate starting the colonization of the substrate. Most of *Aspergillus* species are saprophytes and soil inhabitants. Human and animal foods are ideal organic substrates in which, thanks to their enzymatic activity, they can grow degrading chemical components like hemicellulose, celluloses, pectins, but also fats and oils. They can contaminate major agricultural commodities before or after harvest, causing decay in storage or disease in plants. Although acid pH and low amounts of water normally do not support fungal growth, most of *Aspergillus* species are able to grow at these conditions, colonizing foods and nuts [35].

During the last decades of '900 the discovery of toxins produced by *Aspergillus* associated with poultry and other domesticated animals' deaths all over the world, raised new awareness that *Aspergillus* were very dangerous for both human and animal health [36, 37].

There are 4 groups of aflatoxins (AFs): aflatoxins B1 and B2, aflatoxins G1 and G2. Other mycotoxins, as M1 and M2, originating from their metabolism in humans and animals, can be found in milk and dairy products [38]. Aflatoxins are the most important and dangeroustoxins produced by *Aspergillus* in food, feed and nuts, especially in peanuts. They are produced mostly by some strains of *A. flavus* and *A. parasiticus*. In recent years other species were classified as aflatoxigenic, like *A. bombycis*, *A. ochraceoroseus*, *A. nomius* and *A. pseudotamari*, but compared to the first two mentioned, they are found less frequently in foodstuffs [39].

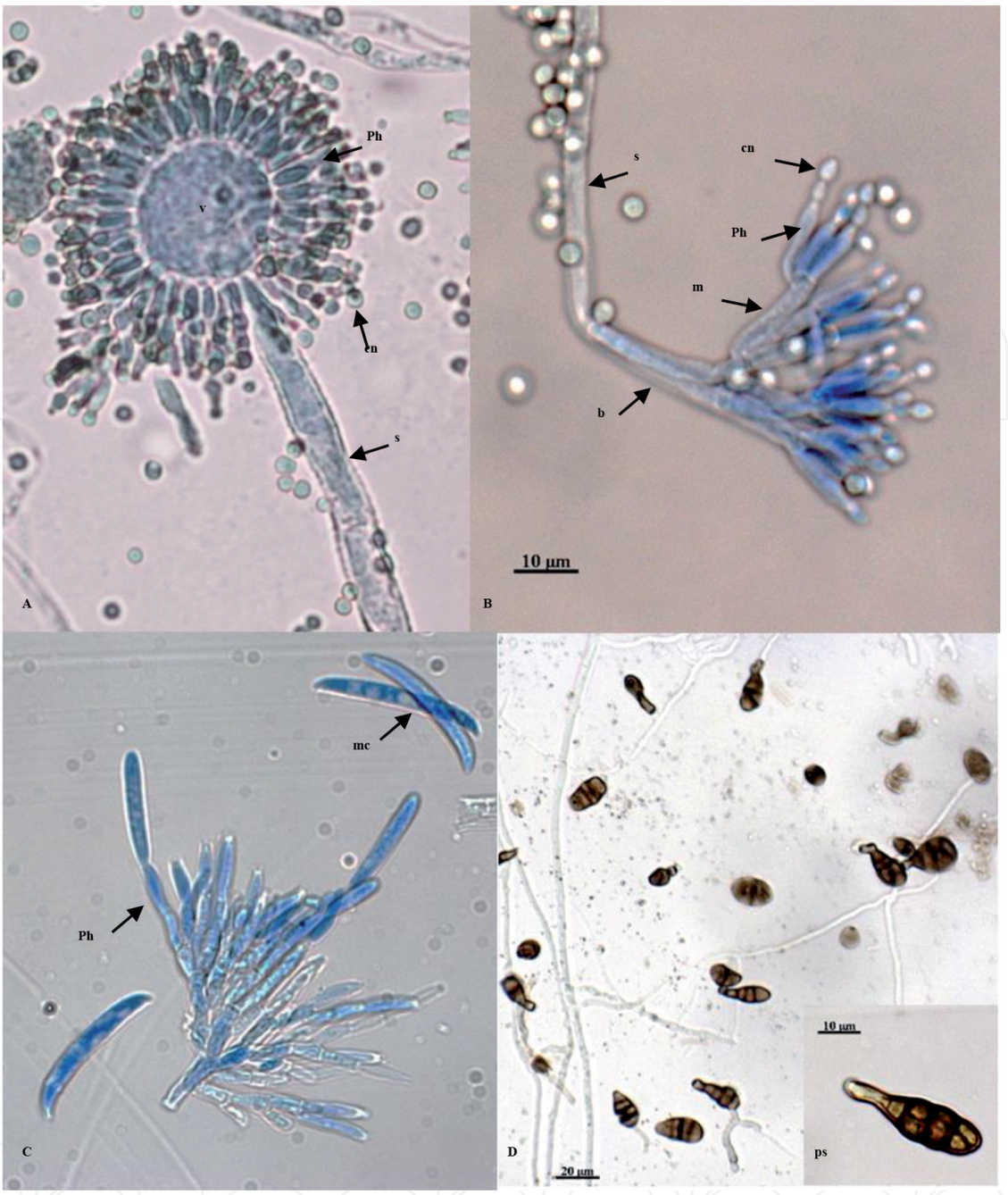

Figure 1.
Microscopic characteristics of Aspergillus *sp. (A),* Penicillium *sp. (B),* Fusarium *sp. (C),* Alternaria *sp. (D). cn = conidia; Ph = phialides; v = vesicle; m = metulae, s = stipe; b = branch; mc = macroconidia; ps = pluriseptate conidium of* Alternaria *sp. Scale bar: A-C = 10 µm; D = 20 µm.*

Other secondary toxic metabolites produced by *Aspergillus* species and in particular by *A. niger* and *A. ochraceus*, are ochratoxins. This group includes ochratoxin A (OTA), ochratoxin B (dechlorinated OTA) and ochratoxin C (ethylated OTA). Among them the most studied for its high diffusion and its toxicological importance is OTA. This toxin, has nephrotoxic effects in humans and animals and has carcinogenic action [38, 40].

3.2 The genus *Penicillium*

Penicillium is one of the most common fungi occurring in a wide range of habitats with worldwide distribution and high impact on economy, human and

Figure 2.
Nuts contaminated with Aspergillus flavus *(a),* A. niger *(b),* Penicillium *sp (c),* Fusarium *sp (d).*

animal life. Its name derives from its conidiophore shape, with a brush-like appearance (a penicillus). It belongs to *Aspergillaceae* family and it's divided in 32 sections with almost 500 species; the teleomophs are ascribed in ascomycetes genera, as *Eupenicillium* and *Talaromyces* [30]. In nature, Penicillium species live decomposing organic materials. On the contrary, other species of *Penicillium* are widely used in the food industry for the production of cheeses such as Roquefort and fermented sausages [41].

For *Penicillium* identification the conidiophores are of great taxonomic importance, resulting from the different branching of the stipe. Depending on the species, in fact, there may be only phialides at apex of stipe, producing conidia, metulae and phialidia, or different order of metulae and phialides. In particular, conidiophore shapes range from being simple to quarter-verticillate depending on the branching levels between the stipe and metulae and phialides. Conidia, typically globose, are produced in long chains by phialides and, when mature, show different colors (**Figure 1b**). Macroscopically, colonies appear of velvety consistency, they are fast in growth and with color varying from green to light blue (**Figure 2c**) [41, 42].

Ecologically, *Penicillium* species live as saprophytes showing optimal growth at low and moderate temperature ranging from 5 and 37°C. Some species colonize decaying vegetation, other are specialized in infecting food commodities. *Penicillium* spores are consistently spread by airborne dispersion and contaminate a large variety of organic substrates. Rapid fungal colonization and intense enzymatic activity cause the spoilage of infected materials. *Penicillium* can invade plants before harvest or during storage. They are also capable to grow during drying process, colonizing substrate poor in water, like nuts [43, 44].

In addition to damages caused in fruits and vegetables by *Penicillium* colonization, some species are able to produce a great variety of mycotoxins. These include ochratoxin A, patulin, citrinin, penicillic acid, cyclopiazonic acid, citreoviridin, roquefortine C and other secondary metabolites. Ochratoxin A and patulin are the most studied due to their worldwide diffusion and their implication in animal and human diseases [45]. In particular, exposure to these two toxins results in mutagenic, teratogenic, neurotoxic, genotoxic and nephrotoxic effects or acute effects like nausea and gastrointestinal damages. The major producers of ochratoxin A in nature are *P. verrucosum* and *P. nordicum*, while *P. expansum* is the major producer of patulin [46]. While patulin is commonly detected in fresh fruits like apple and its derivates, ochratoxin A can be found in a large number of food products including nuts [47].

3.3 The genus *Fusarium*

The anamorphic fungi belonging to genus *Fusarium* are ubiquitous, growing in soils but also in living or dead plants and in several commodities. These ascomycetes, ascribed to *Hypocreaceae* family and mainly included in the genus *Giberella*, currently contains several complexes species. Some of them, in particular those contaminating commodities, produce several mycotoxins with acute and chronic effects on human and animal health [48, 49].

Microscopic characteristics such as macro-, microconidia and chlamydospores of *Fusarium* are distinctive for genus identification. Macroconidia, produced from phialides sometimes gathered in sporodochia, are long, slender, dorso-ventrally curved (spindle-shaped), pluricellular and with a basal foot cell near the attachment point to the phialide (**Figure 1c**). Is this particular shape of macroconidia that defines the genus. Microconidia, formed from aerial mycelium are little propagule, mono or bicellular, smooth, hyaline and ovoid or cylindrical in shapes. Chlamydospores are overwintering structures, rounded, produced vegetatively from mycelium, able to resist to unfavorable conditions and germinate under the onset of suitable conditions. Macroscopically, colonies are wooly or cottony, flat and presents several colors such as white, cream, tan, salmon, cinnamon, violet, purple (**Figure 2d**) [50, 51].

Fungi of the genus *Fusarium* generally prefer cool climates and have a complex ecology. As saprotrophs, they live in the soil, on crops or on decaying organic material. Many species are primary pathogens in field, capable of causing vascular diseases. The most affected crops in the field are wheat and corn, but infection by these fungi can also occur in vegetables, nuts, ornamental plants, trees and foodstuffs during post-harvest storage [7, 52, 53]. Some *Fusarium* species produce dangerous mycotoxins that cause cellular toxicity, effects on animal growth and development and human cancer [54]. The main toxic classes produced by *Fusarium* include trichothecenes such as T-2 toxin, deoxynivalenol (DON) and nivalenol (NIV) produced mainly from *F. sporotrichoioides*, *F. graminearum* and *F. culmorum*; fumonisins B and B2 produced mainly from *F. fujikuroi* and *F. proliferatum*; zearalenone produced from *F. culmorum*, *F. graminearum* and *F. crookwellense* [55].

3.4 The genus *Alternaria*

The genus *Alternaria* belongs to the Ascomycota family of *Pleosporaceae* and it is divided into 26 section and approximately 300 species (*Lewia* is the most known sexual stage), characterized by saprophytic and pathogenic species causing plants diseases, postharvest damages and humans' allergies [56]. Some species of *Alternaria* also produce several mycotoxins in infected plants and foods [57].

Macroscopically, the colonies are flat, downy to wooly and the surface is grayish-with at the beginning and greenish-black or olive-brown at maturity [58].

Characteristics of *Alternaria* is the production in chains of dark-colored and multi-celled conidia ovoid to oblcavate, with longitudinal and transverse septa and a beak tapering apical cells (**Figure 1d**) [59, 60].

Mycotoxigenic fungi	Nuts	References
Aflatoxigenic fungi		
Aspergillus flavus	Almonds	[65–69]
	Hazelnut	[65, 66, 70, 71]
	Peanuts	[65, 66, 69, 72–74]
	Pistachios	[65, 66, 72, 74, 75]
	Walnuts	[11, 65, 66, 69, 72, 76–78]
A. parasiticus	Hazelnut	[70]
	Walnuts	[78]
Ochratoxigenic fungi		
A. ochraceus/*A niger*	Almond	[79]
	Chestnut	[80]
	Hazelnut	[74]
	Peanuts	[72, 73]
	Pistachio	[69, 72, 74]
	Walnuts	[69, 74–77]
Fusarium		
Fusarium spp.	Almonds	[65, 81, 82]
Fusarium spp.	Chestnuts	[81]
Fusarium spp.	Hazelnuts	[65, 74, 79, 83, 84]
F. sporotrchioides		
Fusarium spp.	Peanuts	[65, 66, 73, 83]
F. reticulatum		
F. sambucinum		
Fusarium spp.	Pistachio	[83]
Fusarium spp.	Walnuts	[65, 75, 78]
F. solani		
F. culmorum		
F.oxysporum		
Alternaria		
Alternaria spp.	Almonds	[81]
Alternaria spp.	Chestnuts	[81]
A. alternata	Walnuts	[75, 76]
A. atrans		
A. quercus		
A. tenuissima		

Table 1.
Most recurrent toxigenic fungi isolated from some nuts reported in recent studies.

Alternaria spp. colonize a wide range of plants and growth as saprophytes in plant residues, in soil or as fungal pathogens, colonizing mostly fruits and herbaceous plants and it is diffused in humid environment characterized by temperature ranging from 18 and 32°C [61]. *Alternaria* spp. can produce toxic metabolites that play a fundamental role in fungal pathogenicity and food safety. Today are known about 70 alternaria-toxins, some of them very dangerous for humans and animals [62], including alternaiol, alternariol monomethyl ether and tentoxin commonly founded in substrates like tomato, oil seeds, wheat, blueberries and walnuts. Toxicological data about mycotoxins produced by *Alternaria* are very limited but they have been shown to have cytotoxic, fetotoxic and teratogenic effect on animals [63]. Recent studies focused on emerging groups of mycotoxins produced by *Alternaria* species, described as potentially hazardous [64].

All the fungi mentioned above were frequently isolated from nuts all over the world (**Table 1**).

4. Detection methods of contaminating fungi and mycotoxins in nuts

In the last decades several laboratory methodologies were defined both to detect, enumerate and isolate fungi from nuts and to evaluate the presence of single specific mycotoxins and their respective concentrations. However, so far, only few methods can be considerate "official" and validate by Public Authority.

To apply isolation techniques, is necessary to take a representative sample to submit to the laboratory test and, also for this, the official regulations provide the correct methods of sampling, handling and pretreatment of the samples.

To detect, enumerate and isolate contaminating fungi from sampled nuts two cultural-based methods are most frequently applied: the dilution plating [85] and the direct plating [86]. In the first case, unshelled or shelled nuts are finely chopped or homogenized in a mixer and suspended in water or water containing 0.1% peptone. Serial decimal dilutions are spread on agarized nutrient medium in Petri dishes (plates). In the second case, nuts particles (or whole seeds) are placed directly on media, eventually after surface sterilization in sodium hypochlorite.

The agarized artificial nutrient media most employed are:

PDA (Potato Dextrose Agar), also known as the "universal substrate", it is mainly used to stimulate the growth and sporulation of filamentous fungi;

SDA (Sabouraud Dextrose Agar) is the medium normally used for the primary isolation of fungi, often with the addition of antibiotics. This medium allows the detection of the "standard" morphology, limiting the development and sporulation of fungal colonies;

DG18 (dichloran-glycerol agar), recommended for the evaluation of CFU and the isolation of yeasts and molds from dry and semi-dried foods, including fruits, spices, grains, nuts, meat and fish products [85];

DRBC (Agar Dichloran Rosa Bengal Chloramphenicol), promotes the selective growth of molds and yeasts present in food [87];

PCA (Agar Plate Count) is an agar medium that allows the non-selective growth of molds, yeasts and bacteria;

OGYE AGAR BASE, contains yeast extract, useful for the growth and UFC count of molds in clinical, food and dairy product samples.

Media recommended for *Aspergillus* and *Penicillium* identification include Czapek Yeast Autolysate agar (CYA) and Malt Extract agar [34, 41]. More characters useful for other taxonomic characters can be obtained by using other media such as

Czapek's agar (CZ), Yeast Extract Sucrose agar (YES), Oatmeal agar (OA), Creatine Sucrose agar (CREA), Dichloran 18% Glycerol agar (DG18), Blakeslee's MEA and CYA with 5% NaCl [34, 41].

Specifikke nutrient-arme agar (SNA), potato dextrose agar (PDA) and YES agar can be used for *Fusarium* identification while dichloran rose Bengal yeast extract sucrose (DRYES) agar and potato carrot agar (PCA) are suggested for *Alternaria* species [88, 89].

All plates are incubated at temperatures ranging from 25 and 30°C for up to 7–9 days and, every 3 days, observations are made on the number and type of fungal colonies grown. Higher incubation temperatures are useful to distinguish between species [34, 41].

In the methodology of the dilution plating the total number of fungal colonies (colony-forming units, CFU), referred to the relative decimal dilution, is used to calculate the level of fungal contamination of the analyzed sample. Moreover, observations under the stereoscopic microscope and the optical microscope allow to identify the genus of belonging of the colonies. It is therefore possible, on the total of the CFUs detected, to determine the percentage of the different mycotoxinogenic genera.

In direct plating analyses, results are usually expressed as percentage of infected particles/nuts.

Among the grown fungal colonies, some of the most representative, because probably belonging to mycotoxigenic species (yellow and black aspergillia, green and blue penicilli, *Fusarium* spp., etc.) are first transferred into plate containing agarized medium and purified to be submitted to identification tests and other analysis.

Although culture-based methods for detection and identification of fungal toxin producing fungi are widely used, they showed some limitation due to time-consuming and labour-intensive aspects. It also requires facilities and mycological expertise.

For this reason polyphasic approach based on morphological, physiological and molecular methodologies is suggested for a more accurate identification of mycotoxin fungi producer [89]. Morphological analysis, macro and microscopic observations are made, also growing the isolate on suitable media and at different temperatures. Macroscopic features such as shape, color, texture and speedy growth of the colonies, among others, and microscopic characteristics as mycelial structures, spores, conidia are often fundamental taxonomic characters to identify the species [31, 34, 41, 90–92].

It is not uncommon, however, that these characters are not sufficient for exact identification. In order to avoid mistakes it is possible to consider some physiological parameters (enzymatic activities, secondary metabolites production, etc.) that can provide further data for better identification [93]. It is always good practice, in any case, to support and confirm the identification of the fungal isolate with appropriate DNA based molecular analyzes. A DNA marker for reliable species identification is the internal transcribed spacer rDNA area (ITS) now accepted as the official barcode for fungi [94]. However, this locus is insufficient for correctly identifying some species and other possible secondary markers include 'nuclear large ribosomal subunit' (LSU rDNA) [95], the 'nuclear small ribosomal subunit' (SSU rDNA) [96], 'β-tubulin (BenA)' [97], 'elongation factor 1-α (EF-1-α)' [98] and the 'second largest subunit of RNA polymerase II (RPB2)' [99].

The identification of fungi using molecular markers is improved after DNA isolation from mycelium of pure and axenic cultures [100]. A great variety of DNA extraction methods are available [101]. Commercial kits and customized methods are typically employed. Critical to the successful isolation of nucleic

acids is the cell disruption step with an appropriate buffer (typically CTAB) and techniques that assure high quantity and quality of nucleic acids and no release of potential PCR inhibitors. After this step, nucleic acids can be purified to eliminate impurities [102]. Species-specific PCR methodologies targeting conserved genes or regions of taxonomical interest or by focusing on the mycotoxigenic genes have been extensively applied for identification of mycotoxigenic fungal contaminants [103–106].

In order to detect and quantify mycotoxins in nuts there are several analytical techniques, but official controls are carried out with screening methods, using immunoenzymatic techniques (ELISA) and with confirmatory methods as high-performance liquid chromatography with fluorescence detection (HPLC-FLD) coupled to tandem mass spectrometry (LC–MS/MS). In most cases, methods are validated on a single matrix and do not meet the supervisory bodies' needs that require different mycotoxin limits for different matrices. Regarding extraction method, solid-phase extraction (SPE), solid–liquid extraction (SLE), and liquid–liquid extraction (LLE) are common techniques used for LC–MS/MS analysis for mycotoxin. The challenge of mycotoxin analyses is that they have different proprieties and polarity. Therefore, the right choice of the extraction method can be difficult.

More recently new techniques were developed to detect low-level mycotoxin contamination, to reveal the presence of "masked mycotoxins", complex food matrices in which the mycotoxin contamination occurs or to evaluate the co-occurrence of more mycotoxins in the same sample [6].

5. Strategies to limit contaminating fungi and mycotoxins in nuts industrial chain

Fungal and mycotoxins contamination occurs both in field (pre-harvest), during harvesting and post-harvest management [107]. In particular, aflatoxigenic aspergilli are of significant concern in terms of consumer health.

Generally, in field, several factors not easily controllable, can have a significant impact on fungal infection, including: crops damages due to drought or to insect, delay in harvesting, extreme weather events such as heavy rains, and or sudden frosts. Moreover, it is well known that any management practice to maximize plant performance and decrease plant stresses will decrease fungal and AFs contamination. Proper agronomic practices (tillage, fertilization regimes, right plant density and irrigation), guaranteeing the best vegetative development of dried fruit plants, can represent a valid means of prevention. Different cultivars of nuts show different susceptibility to *A. flavus* and AF accumulation. The conversion of orchards into more resistant cultivars is one possible measure of control [108–111].

Moreover, control of parasitic insects or other biotic adversities can limit the development of contaminating fungi, able to settle in the fruits through the lesions they cause [112].

During the harvesting process, great care must be taken to maintain the integrity of the product, avoiding contact with the ground or with other materials with a high risk of contamination. The damaged products are certainly those most susceptible to contamination during the subsequent phases.

Transport and storage are two important stages to be monitored [68]. Drying should take place soon after harvest and as rapidly as possible, because the prolongation of temperature, humidity and ventilation conditions favorable to the development of microorganisms, can lead to irreversible contamination of the product by both fungi and mycotoxins.

Nuts differ in their storage requirements in function of oil and fatty acid compositions. Temperatures ranging from 4 to 15°C, kernel moisture content around 2.5%, a_w of about 0.7, or relative humidity below 80%, oxygen concentration below 2.5%, and dark conditions are ideal storage conditions for most tree nuts [107, 113].

In post-harvest some physical and chemical methods aimed at decontamination from aflatoxins can be effective for their control. Removal of visibly damaged nuts by manual or mechanical sorting prior to processing significantly reduces AF contamination, limiting the number of potentially contaminated nuts in subsequent processing steps [114]. Other processes, although not very convenient from a practical point of view, can allow to reduce the concentration of AF, such as chemical (ammonia) or thermal (peanut roasting) treatments. Some other technologies, such as irradiation and improved packaging materials, can also minimize post-harvest aflatoxin contamination [115].

However, the best solutions to reduce aflatoxin contamination and improve both economic sustainability and food safety, are the integration of pre- and post-harvest technologies.

In order to protect the health of consumers and ensure the fairness of international trade, FAO and WHO have developed a code of good practices to help contain the phenomenon (www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXC%2B59-2005%252FCXP_059e.pdf).

These practices are also applied to all nuts and are summarized in the following points:

- early harvest of the fruit, preferably manual in order to reduce mechanical damage and avoid contact with the ground;
- in the post-harvest period, select the product and dry it quickly, reducing the level of humidity and the possibility of fungal growth;
- maintain proper ventilation during product storage, so that the fungal population and the concentration of aflatoxins are as low as possible or better still non-existent;
- thoroughly clean and sanitize the premises to reduce the risk of contamination.

In addition to these practices, some studies have highlighted the possibility of using biological control strategies for the control of mycotoxinogenic fungi [112].

The definition of programs to monitor fungal and mycotoxin contamination in processed nuts are essential to ensure the food safety for the consumers and an effective scientific system for the control of mycotoxin levels is represented by the HACCP method (Hazard Analysis and Critical Control Point).

This method is based on the preventive analysis of hazards and on the control of so-called critical points detected during the production and manufacturing process [116].

The HACCP strategy is quite simple, at least in theory, as it considers all the steps of the supply chain, from the choice of the cultivars to the finished product ready for marketing and defines the control protocols on each phase of the process.

6. A study case: fungal contaminants and mycotoxins in almonds and pistachios in Sicily (Italy)

In last years (2016–2021) at the Experimental Zooprophylactic Institute of Sicily (Palermo, Italy), analyzes on contamination by total aflatoxins or AF B1 have been

carried out on 618 samples of pistachios, from both Sicilian and foreign origin, using the validate HPLC method coupled with fluorescence detection (FLD). Out of this large numbers of controls, only 7 samples were positive for total aflatoxins, whose contamination ranges were included between the values 0.2 and 1.7 micrograms per kg, lower than those required by European regulations.

In order to acquire information also on the state of contamination by mycotoxinogenic fungi, an investigation aimed at the isolation and identification of any fungal microorganisms present in both pistachio and almond samples was recently carried out. The samples were analyzed at the laboratories of the Department of Agricultural, Food and Forestry Sciences of the University of Palermo, for mycological tests and at the Experimental Zooprophylactic Institute of Sicily, for toxicological tests.

Shelled almond and pistachio samples were collected from different warehouses located in Southern Italy and analyzed in order to determinate total fungal contamination and the frequencies of toxigenic genera (*Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria*). Isolation and enumeration of fungal colonies were carried out employing the dilution plating methodology [85] on PDA. All plates were incubated at 25±1°C for 9 days and the total fungal contamination was evaluated every 3 days. *Aspergillus*-like colonies found growing in the isolation plates were subcultured into PDA and monoconidial pure cultures were assigned to their corresponding section or species based on macroscopic and microscopic characteristics.

The macro-morphological features recorded were colonies diameter, color, shape and texture. Microscopic observations were performed using an Axioskop (Zeiss, Oberkochen, Germany) microscope coupled to an AxioCam MRc5 (Zeiss, Oberkochen, Germany) digital camera. The microscopic characteristics recorded were dimension of conidial heads, vesicle shape and diameter, presence of metulae, size and shape of phialides and conidia. Finally, morphological identification was carried out using taxonomic keys.

Although the toxicological tests on the samples of nine walnuts excluded the presence of aflatoxins, first results showed that fungal contamination in almond and pistachio samples ranged from 2.5x10 to $2.6x10^4$ and 2.5x10 to $1.5x10^3$ CFU/g respectively. The genera *Aspergillus* and *Penicillium* were recovered at high frequencies both in the pistachio and almond samples with significantly differences within the samples. Occasionally, other fungal genera were isolates (*Mucor*, *Rhizopus*, *Paecilomyces*, etc.), while no *Alternaria* or *Fusarium* colonies were observed. *Aspergillus* was the most predominant fungal genus both in almond (77%) and pistachio (89.8%) samples. *Penicillium* spp. frequencies ranged from <1 to 15.9%. These data are comparable to those reported in similar studies both in relation to the level of total fungal contamination an as regard the diffusion of the two considered genera [72, 74].

According to morphological characteristics, *A. flavus* and *A. niger* were identified in pistachio samples, whereas no *A. flavus* was isolated from almonds samples.

7. Conclusion

The problem of mycotoxins has become one of the aspects that most affect the nuts market, even if the real problem is represented by mycotoxinogenic fungi that can contaminate these commodities. Fungi infecting nuts in field can rapidly grow and produce mycotoxins during storage when conditions are suitable. Preventing AFs and other mycotoxins accumulation in the finished product can be achieved by either controlling the contaminating fungi or mycotoxins production in pre- or

post-harvest stages of production, by using any of several measures alone or in combination [108–111].

However, the more articulated and complex the production chain is, the greater is the risks and possibilities of contamination by fungi and their mycotoxins. Furthermore, although the concentration of the most dangerous aflatoxins may be very low or completely absent, some mycotoxigenic fungi may be present and in favorable condition can produce these secondary metabolites making the food no longer safe for the consumer.

In order to guarantee, products healthiness, mycotoxins detection should be supported by mycological analysis throughout the entire supply chain. Another appropriate practice should be to investigate the presence of a greater number of mycotoxins, given the great biodiversity of fungi potentially capable of contaminating nuts.

Several guidelines, therefore, have been developed to identify critical points in the production process and to define strategies aimed at directly and indirectly reducing the production and spread of mycotoxins.

Contamination prevention is necessary because most detoxification methods require testing and are not completely approved by the industry or follow all safety requirements. Quality assurance systems for tree nut industries, including supplier qualification, are essential to prevent the presence of toxigenic fungi and prevent the consequent production of toxins along the production chain.

Moreover, there is a significant interest in considering the impact of climate changes on mycotoxin-producing fungi during pre- and post-harvesting [117].

Author details

Giulia Mirabile[1], Patrizia Bella[1], Antonio Vella[2], Vincenzo Ferrantelli[2] and Livio Torta[1*]

1 Department of Agricultural, Food and Forestry Sciences, University of Palermo, Palermo, Italy

2 Experimental Zooprophylactic Institute of Sicily, Palermo, Italy

*Address all correspondence to: livio.torta@unipa.it

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