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Filamentous Fungi and Mycotoxins in Cheese: A Review

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Abstract: Important fungi growing on cheese include *Penicillium*, *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor*, and *Trichoderma*. For some cheeses, such as Camembert, Roquefort, molds are intentionally added. However, some contaminating or technological fungal species have the potential to produce undesirable metabolites such as mycotoxins. The most hazardous mycotoxins found in cheese, ochratoxin A and aflatoxin M1, are produced by unwanted fungal species either *via* direct cheese contamination or indirect milk contamination (animal feed contamination), respectively. To date, no human food poisoning cases have been associated with contaminated cheese consumption. However, although some studies state that cheese is an unfavorable matrix for mycotoxin production; these metabolites are actually detected in cheeses at various concentrations. In this context, questions can be raised concerning mycotoxin production in cheese, the biotic and abiotic factors influencing their production, mycotoxin relative toxicity as well as the methods used for detection and quantification. This review emphasizes future challenges that need to be addressed by the scientific community, fungal culture manufacturers, and artisanal and industrial cheese producers.

Keywords: Mold, filamentous fungi, mycotoxins, cheeses, diversity, mycotoxin toxicity, mycotoxin biosynthesis, mycotoxin ecological role, mycotoxin control

Introduction

Many food spoilage fungi belonging to the genera *Aspergillus*, *Fusarium,* and *Penicillium* produce mycotoxins that are toxic for vertebrate and other animal groups when introduced via a natural route (food ingestion). Naturally present in ambient air, soil, and crops (Yiannikouris and Jouany 2002), mycotoxigenic fungi are considered to be among the most significant contaminants in foods in terms of impact on public health, food safety, and the national economy of several countries (Steyn 1995; Pitt 2000).

The term mycotoxin, derived from the Greek words "*mykes*" meaning fungi or slime molds and "*toxicum*" referring to toxin, was utilized for the first time in 1962 following a veterinary crisis that resulted in the death of about 100000 young turkeys. These birds had ingested contaminated peanut meal containing secondary metabolites (aflatoxins [AFs]) produced by *Aspergillus flavus* (Blount 1961; Hundley 2001).

Mycotoxins are defined as secondary metabolites, hence not directly essential for normal fungal growth, and are often low-molecular-weight compounds. They are naturally occurring molecules and are thought to confer a selective advantage to the producer strain within complex ecosystems. Their role is to communicate and compete with other organisms or to inhibit competitor growth and reproduction in the same trophic niche (Magan and Aldred 2007; Fox and Howlett 2008). Early mycotoxin production could also allow molds to rapidly colonize the environment. These metabolites are commonly found in various food and feed commodities. Indeed, molds are able to grow on a wide range of substrates (including cereals, meats, nuts, cheeses, grapes, coffee beans, apples, and derived products), at any stage of production in the field, during postharvest storage, and under a wide range of climatic conditions. Moreover, mycotoxins are known to be resistant to industrial processing. It has been established that mycotoxin production profiles may change under different fungal growth conditions including substrate composition (Kokkonen and others 2005a), ecophysiological factors including temperature, water activity, pH or oxygen concentration, and biotic factors including microbial interactions (Magan and Aldred 2007).

Analytical methods for identifying and quantifying mycotoxins in foods and feeds have been widely developed. Maximum levels for AFs, deoxynivalenol, fumonisins, ochratoxin A (OTA), patulin, T2 and HT-2 toxins, and zearalenone are regulated in the EU (Commission Regulation [EC] Nr. 1881/2006) as well as worldwide due to their well-characterized adverse health effects after acute, short-term, and/or long-term exposure. In cheese, aflatoxin M1 (AFM1) is the only mycotoxin for which maximum levels (0.05 and 0.5 ppb in the milk used for cheese-making in the EU and United States and China, respectively) have been set.

In cheese, the most hazardous mycotoxins are OTA and AFM1. They are, respectively, produced by unwanted fungal species either via direct cheese contamination (spoilage agents) or indirect contamination of the milk used for cheese-making due to feed contamination. Among fungal cheese spoilers, the most problematic

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mycotoxigenic ones belong to *Penicillium* spp. as they are particularly well adapted for growth on and/or in the cheese matrix. Filamentous fungi also play a central role during cheese-making. Two well-known fungal ripening cultures, *Penicillium camemberti* and *Penicillium roqueforti,* which play a significant role in appearance, texture, and flavor development of mold-ripened and blue-veined cheeses, are also known for their ability to produce several mycotoxins. For example, *P. camemberti* produces cyclopiazonic acid (CPA), while roquefortine C and mycophenolic acid (MPA) are produced by *P. roqueforti*. Yet, these metabolites exhibit lower toxicity than those reported above and are present at low levels in cheese. Due to their long history of safe use in cheese in the U.S.A. and EU (no cases of food poisoning associated with the consumption of contaminated cheese were described in literature), use of these fungal cultures is regarded as safe in their intended conditions of use (cheese-making). In addition, these microorganisms are granted a generally recognized as safe (GRAS) status by the U.S Food and Drug Administration (FDA). However, in the EU, while the European Food Safety Agency (EFSA) acknowledged that there are no reports of adverse health effects for cheeses produced with these microorganisms, the qualified presumption of safety (QPS) status was not granted for these species. This was mainly due to a lack of knowledge on mycotoxin production by the strains intentionally used, the occurrence of these mycotoxins in cheese and their toxicity (EFSA 2011). In this context, scientific questions remain concerning the presence of mycotoxins in cheese, their variability in terms of quality and quantity, relative toxicity levels, impact of biotic and abiotic factors on their production as well as their control.

The aim of the review is to summarize the current knowledge on filamentous fungi and mycotoxins in cheese. The paper deals with fungal diversity, the main mycotoxins reported in cheeses, mycotoxin toxicity, and their detection and quantification. The biosynthetic pathways described to date in cheese-related species as well as abiotic and biotic factors impacting mycotoxin production in cheese are also presented. Finally, physical, chemical, and biological methods potentially usable to prevent fungal growth and indirectly eliminate or reduce mycotoxin levels in cheese are discussed (from farm to fork).

Filamentous Fungi in Cheeses: The Good and the Bad

Few studies have extensively investigated fungal communities in cheeses. Barrios and others (1998) provided a global view of mold diversity in 52 commercial cheese susing a culture-dependent approach. *Penicillium* species were the most frequent (found in 63% of cheeses), followed by *Mucor* spp. (27%), *Geotrichum candidum* (17%), and 12 other fungal genera identified in 2 to 10% of the samples. Such culture-dependent methods have also identified *Penicillium* spp. as the prominent mold in sheep and goat cheeses from different origins (Montagna and others 2004; Hayaloglu and Kirbag 2007; Båth and others 2012). More recently, culture-independent methods have allowed to easily evaluate fungal (both undesirable and technological) community dynamics in cheese (Jany and Barbier 2008). In this context, Flórez and Mayo (2006) used PCR-DGGE to study the dynamics of Cabrales blue-veined cheese microbial communities. In artisanal Cabrales cheese, they found a fungal community composed of *P. roqueforti*, *P. chrysogenum,* or *P. griseofulvum* that did not change during ripening. Concerning mold surface-ripened cheeses, Arteau and Labrie (2010) recently studied the fungal communities of Camembert and Brie cheeses using a culture-independent T-RFLP method. In this study, *Cladosporium cladosporioides*, *G. candidum*, *M. racemosus*, *P. camemberti*, *P. caseicola*, *P. chrysogenum*, *P. commune,* and *P. roqueforti* filamentous fungal species were observed. Finally, use of both culture-dependent and culture-independent methods allowed Panelli and others (2012) to reveal the prominence of *P. commune* over other fungal species such as *Cladosporium* spp. in Taleggio cheese, whereas in Fossa cheese, the fungal community was dominated by *Penicillium* and *Aspergillus* species (De Santi and others 2010).

Microorganisms play a central role during cheese-making, as for other fermented products. When the primary microbiota involved in acid production is mainly composed of starter lactic acid bacteria (LAB), the secondary microbiota includes salt-tolerant bacteria, yeasts, and filamentous fungi that mainly contribute to organoleptic properties of cheeses. The role of filamentous fungi is particularly important for both surface mold-ripened and core mold-ripened cheeses. Indeed, functional features and degradation of milk constituents, such as proteins and lipids, can lead to texture, flavor, and nutritional cheese quality improvement (Fox and McSweeney 2004). Among the species encountered in cheeses, *P. camemberti*, *P. roqueforti*, *Mucor fuscus*, *M. lanceolatus*, *G. candidum*, *Fusarium domesticum*, *Sporendonema casei*, *Scopulariopsis flava,* and *S. fusca* are the most common filamentous fungi used as ripening cultures (Hermet and others 2012; Ropars and others 2012).

Filamentous fungi encountered in cheeses may originate from raw materials such as milk or may be introduced during cheese-making either from the environment or are deliberately inoculated using commercial ripening cultures. Delavenne and others (2011) showed that cow milk samples contained high fungal diversity with up to 15 species in a single sample, whereas a maximum of 4 or 6 different species were recovered in goat and sheep milks, respectively. These authors identified fungi belonging to the following genera: *Aspergillus*, *Chrysosporium*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Torrubiella*. Vacheyrou and others (2011) concluded that most fungal species found in cow milk originated from a transfer from the stable to the milking parl and then to milk. Fungi occurring in raw milk or in the cheese-making environment are not very likely to persist and/or grow in cheese due to the biochemical composition of cheese and the hurdle effect exerted by the cheese microbiota. Industrial processes may also select certain fungal strains/species resulting in a fungal community shift. Moreover, raw milk heat treatment is often performed prior to manufacturing and eliminates most fungi in cheeses where the presence of molds is undesirable. Filamentous fungi contamination can also be detrimental to cheese quality, causing appearance defects, off-flav, and potentially toxic secondary metabolite production.

P. commune, *P. palitans*, *P. nalgiovense*, and *P. verrucosum* species are the most frequent contaminants as suggested by Lund and others (1995) in a study based on spoilage microbiota of hard, semi-hard, and soft cheeses. Reports of *P. verrucosum* occurrence should, however, be treated with care since a high number of *P. verrucosum* isolates were reclassified as *P. nordicum* a species closely related to *P. verrucosum* that is much more adapted to salt and protein-rich substrates than *P. nordicum* (Larsen and others 2001). Fungal species cannot always be classified as either technological or contaminant species, since the same species can be used as ripening culture in some cases and be undesirable in other contexts (for example, *P. roqueforti* is the major ripening culture in blue-veined cheeses, whereas it is a contaminant in other cheeses). In some cases, the positive (technological species) or negative (spoilage organism or putative mycotoxin producer) role is not always clear and has to be investigated such as in the case of *P. commune* in Taleggio cheeses (Panelli and others 2012).

In the near future, new high-throughput methodologies (such as pyrosequencing) will certainly provide better insight into cheese fungal communities. Among various filamentous fungi

Table 1–Examples of mycotoxins found in cheeses including blue mold cheese. ND: Not determined.

Cheeses	Mold species	Secondary metabolites	Technical analysis	Range	References	Comments
Blue	*P. roqueforti*	Roquefortine, PR toxin, Isofumigaclavine A	Thin-layer chromatography	3.4 μg/g, ND, ND	Scott and others (1977); Lund and others (1995)	
Blue	*P. roqueforti*	100% penicillic acid, 37% PR toxin, patulin	Thin-layer chromatography	ND, ND	van Egmond (1983)	
Blue-molded	*P. roqueforti*	Mycophenolic acid	Thin-layer chromatography	90% 1 to 5 mg/kg	Lafont and others (1979)	
Blue-molded tulum cheese	*P. roqueforti*	Patulin, penicillic acid, PR toxin, roquefortine	Thin-layer chromatography	ND, ND, ND, 2.1 to 3.8 mg/kg	Erdogan and Sert (2004)	
Blue veined	*P. roqueforti*	Penicillic acid	Thin-layer chromatography	ND	Moubasher and others (1978)	Production of penicillic acid is to be due to *P. carneum* (Boysen and others 1996)
Blue		OTA	LC-ESI-MS/MS	0.2 to 0.3 μg/kg	Dall'Asta and others (2008)	
Manchego	*P. roqueforti*	Roquefortine, mycophenolic acid	Thin-layer chromatography	≥30 μg/kg ≥ 20 μg/kg	López-Diaz and others (1996)	
Cheddar	*P. roqueforti*	Penicillic acid, patulin	Thin-layer chromatography	ND, ND	Olivigni and Bullerman (1977)	Production of penicillic acid is to be due to *P. carneum* (Boysen and others 1996) Patulin was the sole metabolite produced by *P. paneum* and *P. carneum* but was not produced by *P. roqueforti* (Nielsen and others 2006)
Tulum cheese	*P. roqueforti*	Penicillic acid, PR toxin, patulin, roquefortine	Thin-layer chromatography	ND, ND, ND	Erdogan and others (2003)	
Tulum cheese		AFM1	LC-MS/MS	11 to 202 ng/kg	Gürses and others (2004)	
Blue veined	*P. roqueforti*	Mycophenolic acid	Thin-layer chromatography	250 to 5000 μg/kg	Engel and others (1982)	
Blue mold	*P. roqueforti*	Roquefortine C Mycophenolic acid	LC-MS/MS	0.0008 to 0.012 μg/kg 0.0003 μg/kg	Kokkonen and others (2005a,2005b)	
Cheese	*P. camemberti*	Cyclopiazonic acid	Thin-layer chromatography	0.5 to 1.5 μg/g	Le Bars (1979)	
Gouda and Edam	*A. versicolor*	Sterigmatocystin	Thin-layer chromatography	5 to 600 μg/kg	Northolt and others (1980)	
Semhard cheese	*Penicillium* sp.	Patulin, ochratoxin	High-performance liquid chromatography	15 to 460 μg/kg and 1 to 262 μg/kg	Pattono and others (2013)	
Turkish cheeses	*Aspergillus* sp.	Aflatoxin M1	ELISA	58 to 850 ng/kg	Aydemir and others (2010)	
Italian white cheeses	*P. camemberti*	Cyclopiazonic acid	High-performance liquid chromatography	0.02 to 0.08 mg/kg	Zambonin and others (2001)	

species encountered in cheese, the mycotoxin-producing ability for some has already been well established, while for others questions still remain.

Mycotoxins in Cheese

In the 1960s, the first studies related to mycotoxins in cheeses were published and led to the discovery of AFs. Other studies were performed in the 1970s with special interest on the occurrence of CPA in cheese. Since 2000 and the development of powerful analytical techniques such as LC-MS/MS, numerous studies have emerged on the subject.

As stated above, various mold species can be isolated from cheeses and correspond to ripening cultures or contaminating molds (Taniwaki and others 2001) that can potentially produce mycotoxins. Analytical methods for identifying and quantifying mycotoxins in foods and feeds have widely been developed and governmental agencies have establishe regulatory limits and guidelines for safe doses.

Various mycotoxins have been detected in cheese (Table 1) and the most commonly identified are described below. Roquefortine C has been quantified in blue cheese (3.4 μg/g) (Scott and others 1977), Manchego cheese (López-Diaz and others 1996), and in commercial Finnish cheeses at levels reaching 12000 μg/kg (Kokkonen and others 2005a, 2005b). MPA has been reported from different types of blue mold cheeses (1.5 mg/kg by Lafont and others1979 and 5 mg/kg by Engel and others 1982) using thin-layer chromatography (TLC) and at much lower levels (0.0003 μg/kg) in cheese using LC-MS-MS (Kokkonen

and others 2005a, b). Due to AFM1 occurrence in milk, this mycotoxin can also be present in cheese. Various studies have reported AFM1 in cheese (Gürses and others 2004; Aydemir and others 2010). On the other hand, very few studies have described AFG1 and AFB1 in cheese. In these cases, the mycotoxins were directly added to cheese and their stability was followed over time. Both AFB1 and AFG1 were stable in Swiss cheese (Lieu and Bullerman 1977). Similar studies were also carried out by inoculating *A. flavus* and *Aspergillus parasiticus* mycotoxinogenic strains in cheese and both mycotoxins were again (Lie and Marth 1967). Sterigmatocystin (STC) was detected and quantified in the surface layer of *A. versicolor* contaminated hard cheeses at levels ranging from 5 and 600 μg/kg (Northolt and others 1980). In white cheese, CPA was detected from 0.5 to 1.5 μg/g (Le Bars 1979) and from 0.02 to 0.08 mg/kg (Zambonin and others 2001). Stott and Bullerman (1976) showed that when patulin was added to cheddar cheese, it was not detected after storage by TLC. However, in semihard cheeses, patulin and ochratoxin were detected at concentrations of 500 and 2620 μg/kg, respectively (Pattono and others 2013). Since PR toxin was discovered (Wei and others 1973), no studies have described this mycotoxin in cheese as it is unstable and leads to PR imine (Scott and Kanhere 1979; Siemens and Zawitowski 1993).

Mycotoxin cheese contamination can occur indirectly via milk contamination or directly by mycotoxin producing spoilage or technological filamentous fungi (O'Brien and others 2004).

Concerning indirect contamination, the main concern is AFM1 in cheese. AFM1 in the final product is due to the addition of this mycotoxin into the milk by dairy animals (Eaton and others 1994; Gallo and others 2008) that have fed on moldy AFB1-contaminated feedstuffs. Indeed, AFB1 is converted into AFM1 by enzymes mainly present in the animal liver and then passed into their urine and milk. Many studies have reported AFM1 in various cheeses (Finoli and others 1983; Taniwaki and van Dender 1992; Gürses and others 2004; Kokkonen and others 2005b). Low storage temperature of cheese can induce a decrease in AFM1 concentration. The indirect contamination of milk with other mycotoxins, such as STC (van Egmond and Paulsch 1986), fumonisins (Maragos and Richard 1994), or CPA (Dorner and others 1994), has also been reported. Concerning this type of contamination in cheese, it is much more complex because it depends on the manufacturing processes used.

Direct mycotoxin contamination can be exogenous *via* the presence of contaminating molds during cheese-making or endogenous by the presence of mycotoxin-producing commercial fungal cultures.

Cheeses on which unintentional mold growth has occurred have been reported to contain mycotoxins. However, mold growth on the cheese surface does not automatically imply that mycotoxins are present in cheese; moreover, mycotoxin production can be quite different among mycotoxicogenic fungi (Moss 1991; Sweeney and Dobson 1998).

In the case of direct contamination from an endogenous origin, several cheeses that intentionally have added mold species during cheese-making for appearance, texture and flavor development can be at risk. Although many cheeses use molds for ripening, the 2 main categories are: soft mold-ripened cheeses (such as "Camembert") associated with *P. camemberti* and blue-veined cheeses (such as "Gorgonzola," "St Nectaire," and "Cantal") associated with *P. roqueforti*. *P. camemberti*, and *P. roqueforti* have long been used in the manufacture of mold-ripened cheeses consumed throughout the world. *P. roqueforti* is an essential component of the microflora of many cheeses including Roquefort (France), Stilton (U.K.), Tulum (Turkey), Gorgonzola (Italy), Cabrales (Spain), Blauschimmelkase (Switzerland), and Danish Blue (Denmark) (Bullerman 1981; O'Brien and others 2004), while *P. camemberti* is associated with Camembert and Brie production. Both species are known for their potential mycotoxin production; however, due to cheese production practices and storage conditions, only low to very low concentrations have been observed (Bullerman 1981; Teuber and Engel 1983).

Overall, numerous mycotoxins can be found in cheeses at highly variable concentrations depending on cheese origin.

Ecological role of mycotoxins in cheese

So far, the ecological roles of mycotoxins have not been completely elucidated but hypothesized functions in natural environments have been proposed. In the cheesemaking context, this role has not been studied to our knowledge. Therefore, ecological role hypotheses in this food matrix can only be proposed based on studies performed in other environments.

Role in competition with other organisms. Mycotoxins are naturally occurring molecules that are thought to confer a selective advantage to the producer within complex ecosystems to communicate and compete with other organisms (Magan and Aldred 2007). Their widespread nature and the preservation of these multigenic biosynthetic pathways suggest that secondary metabolites are used for survival functions in organisms (Demain and Fang 2000). The genes required for the biosynthesis of secondary metabolites are clustered. As stated by Calvo and others (2002), *"logic suggests that their maintenance could only be selected for if the final product conferred some advantage to the producing organism."* In this context, mycotoxins have been proposed to act as chemical signals for communication, a competitive weapon to defend the habitat, or to inhibit the growth and reproduction of competitors of the same trophic niche (Magan and Aldred 2007; Fox and Howlett 2008). The presence of competing microorganisms is essential to mycotoxin production. these hypotheses are demonstrated by the fact that serial transfers on culture media in laboratory conditions (lack of exposure to competitive and natural stressed conditions) result in the loss of AF production in strains of *A. flavus* (Horn and Dorner 2001). Adverse conditions such as micro- and macronutrient depletion and competition with other fungi have a stabilizing effect in preventing AF production loss. In addition, early production of mycotoxins could allow molds to rapidly colonize the environment.

In the cheese ecological niche, both spoilage fungi and fungal cheese cultures are in competition with other microorganisms including bacteria and yeasts. One study showed bacteriostatic effect of roquefortine on gram-positive bacteria, whereas gram-negative bacteria were not influenced at all. This mycotoxin affected bacterial respiration (Kopp-Holtwiesche and Rehm 1990). However, to our best knowledge, the effects of microbial competition on the production of mycotoxins have not yet been studied in cheeses or in cheese-mimicking models.

Role in fitness. Another possible role of mycotoxin production is that it can be related to strain fitness. Mycotoxin production could correspond to a mechanism for adaptation to the environment. For instance, OTA biosynthesis by *P. nordicum* and *P. verrucosum* increases their fitness under high NaCl concentration conditions as exhibited in food products like dry-cured meat or brined olives (Schmidt-Heydt and others 2012). OTA

Table 2–Examples of toxic effects of some mycotoxins produced by *Penicillium* species.

Mycotoxins	Effects	Models	References
Mycophenolic acid	LD50 (Letal Dose) rats 2500 mg/kg IP LD50 rats 500 mg/kg IV LD50 mice 700 mg/kg IP LD50 mice 450 mg/kg IV Mutagenic: chromosomes aberrations, Ames test Immunosuppressive IC_{50} (inhibitory concentration) > 100 μg/mL	*In vivo* rats and mice *In vivo* human Detroit 98 and Girardi Heart *In vitro Salmonella* sp. *In vitro* human macrophage *In vitro* Caco cells	Wilson (1971) Engel and others (1982) Umeda and others (1977); Webner and others (1978) Bentley (2000) Rasmussen and others (2011)
Roquefortine	LD50 rats 1520 mg/kg LD50 mice 169 mg/kg M LD50 mice 184 mg/kg F intoxications Ames test-neurotoxic IC_{50} 48 μg/mL	*In vivo* rats and mice *In vivo* dogs *In vitro Salmonella* sp. *In vivo* day-old cockerel *In vitro* Caco cells	Ueno and others (1978) Scott and others (1977) Puls and Ladyman (1988) Schoch and others (1984) Wagener and others (1980) Rasmussen and others (2011)
PR toxin	LD50 mice 5.8 mg/kg IP LD50 rat 11.6 mg/kg IP LD50 rat 8.2 mg/kg IV Target organs: ↗capillary permeability (heart, liver, lungs, kidneys damages) inhibits RNA and protein synthesis inhibits DNA polymerase mitochondrial respiration mutagenic (Ames+), carcinogenic	*In vivo* mice and rats *In vitro E. coli* *In vitro* rat liver mitochondria *In vitro Salmonella* sp. *In vitro S. cerevisiae*	Chen and others (1982) Moule and others (1976) Wei and others (1984) Ueno and others (1978) Wei and others (1979)
Penicillic acid	Synergistic with OTA	*In vivo* pig	Stoev and others (2001)

contains a chloride atom within its molecule and the constant OTA biosynthesis ensures permanent excretion of chlorine out of the cell for Penicillia (Schmidt-Heydt and others 2012). The more constantly a strain produces OTA, the higher its growth rate will be under high NaCl conditions. Consequently, a strain competes better on a salt-rich habitat in comparison to a strain that produces low amounts of OTA (Schmidt-Heydt and others 2013). Cheese contains a fair amount of NaCl and it is also likely that the NaCl level may influence OTA production in cheeses.

Role in detoxification. Finally, mycotoxin production could correspond to a waste elimination process. Indeed, mycotoxins could contribute to cell detoxification from the accumulation of reactive oxygen species (Reverberi and others 2010; Sanzani and others 2012). Fungi are aerobic organisms producing reactive oxygen species in their environment that can damage cell membranes and metabolic pathways. Generally, a cell can tolerate oxidative stress by increasing antioxidant synthesis and also secondary metabolite production, such as mycotoxins, which are under the control of reactive oxygen species (Reverberi and others 2010). In cheese, most LAB are able to produce hydrogen peroxide (H_2O_2) in the presence of oxygen. Hydrogen peroxide can then be converted to hypothiocyanate (HOSCN) via the reaction of hydrogen peroxide and thiocyanate through catalysis by lactoperoxidase that are both naturally present in milk. These reactive oxygen species, besides the fact that they have known antifungal activities (Schnürer and Magnusson 2005), may also, in turn, affect mycotoxin production in cheese-related fungi. We could also speculate that the production of MPA may be a route for detoxifying acetate, another well-known fungal inhibitor produced, for example, by nonstarter heterofermentative LAB such as *Lactobacillus* spp., however, no scientific evidence yet exists to support that these hypotheses are true.

Mycotoxin production and their biological roles must be considered in an ecological context, which is not easy to elucidate in laboratory conditions. In fact, mycotoxigenic molds do not occur alone, they form part of an ecological niche in which diverse communities of microorganisms compete. A better understanding of the regulation and role of mycotoxin production during cheesemaking could help to develop approaches to control mycotoxin contamination in cheeses.

Nature and toxicity

The usual routes for mycotoxin exposure are ingestion of mycotoxin-contaminated foods and feeds (Hayes 1980; Sweeney and Dobson 1998; Hussein and Brasel 2001; Bennett and Klich 2003), physical contact (CAST 2003), and inhalation (Bunger and others 2004) (Table 2).

Mycotoxins can cause either acute or chronic diseases in animals and humans (Stoloff 1977; Pitt 2000) depending on the dose, ingestion duration, and animal species.

Mycotoxins present in cheese due to fungal contamination.

Aflatoxins. AFs derive from polyketide metabolism and consist of at least 16 structurally related furanocoumarins of which AFB1, AFB2, AFG1, and AFG2 are the most abundant (Huffmann and others 2010). AFs are considered to be among the most significant classes of known mycotoxins in food and feed. They are produced by certain *Aspergillus* species. Highest AF levels are produced as a result of inadequate postharvest storage when high-moisture content and warm temperatures facilitate rapid mold growth. Therefore, the presence of AFs in animal feedstuffs (cereals, forages, and so on) (Garon and others 2006) can be an important contamination source for the dairy industry. As stated above, various studies have reported AFM1 in cheese. Concerning other AFs, AFG1, and AFB1 have been reported in cheese, but only after spiking samples with the mycotoxins or mycotoxinogenic strains (Gürses and others 2004; Aydemir and others 2010).

Lethal dose (LD50) values range from 0.5 to 10 mg/kg according to the considered AF. Animal species respond differently in regard to their susceptibility to chronic and acute AF toxicity. During aflatoxicosis, the liver is the primarily affected organ, leading to bile duct proliferation, centrilobular necrosis, fatty acid infiltration, and hepatic lesions (Heinonen and others 1996; Bhatnagar and others 2002), which ultimately lead to liver cancer (Chao and others 1991; Sweeney and Dobson 1998). Other organs such as the kidney, lung, heart, gizzard, or spleen may also be affected (Yang and others 2012).

AFs induce DNA damage, negatively affect damage repair, and alter DNA base compositions of genes. AF is associated with both toxicity and carcinogenicity in human and animal populations. Within the AF family, IARC (International Agency for Research

on Cancer 1993) has classified AFB1, as a group class 1 proven to cause cancer in human.

Although many different cheese types can contain AFM1, it is almost invariably at such low levels that no danger to human health exists (Blanco and others 1988). But recent studies showed that high AF levels up to 850 ng/kg in regional Turkish cheese (Aydemir and others 2010). Therefore, the health risk should not be underestimated.

Finally, due to AFM1 toxicity (classified by the IARC 2002, as class 2B, possible human carcinogen), many countries tightly monitor AFM1 levels in milk and milk products and, as stated above, the European Union has established a 0.05 μg/kg maximal limit (EFSA 2004).

Sterigmatocystin. STC is a xanthone and is structurally similar to AF (Hamasaki and Hatsuda 1977); however, it is less toxic (Sweeney and Dobson 1998). This mycotoxin is produced by a few *Aspergillus* species (Cole and Cox 1981; Veršilovskis and De Saeger 2010).

STC has indeed been detected in *A. versicolor*-contaminated cheeses (Scott 1989). Northolt and others (1980) reported STC occurrence during cheese (Gouda and Edam) ripening in warehouses in the Netherlands. STC was stable in contaminated cheeses for a 3-mo period at various temperatures (−18 to +16 °C) (van Egmond 1983). However, low temperatures (5 to 7 °C) used during ripening and storage should prevent *A. versicolor* growth and STC production. At these temperatures, molds that develop on cheese rather correspond to *Penicillium* species, which could not produce AFs or STC (Bullerman 1981). Nevertheless, STC was analyzed in cheese samples, produced in Latvia (8) and Belgium (13). Two cheeses (9.5%) were positive for STC with concentrations of 1.23 and 0.52 g/kg, respectively. Five (24%) samples contained STC above the limit of detection (0.03 g/kg) (Veršilovskis and others 2009).

STC is activated to sterigmatocystin-1,2-epoxide by liver enzymes, which binds covalently to DNA and induces various tumors (Baertschi and others 1989; Raney and others 1992). STC produces higher cytotoxicity than AFB1 ($IC_{50} = 10$ μM) in A-549 cultures (lung cell line) (Palanee and others 2001). STC is carcinogenic in animal models and possibly carcinogenic to humans (IARC 1976).

Citrinin. Citrinin can be produced by a dozen *Penicillium* species including a few *P. camemberti* strains and several *Aspergillus* species (Manabe 2001). *P. citrinum*, *P. viridicatum, P. expansum*, *P. verrucosum*, *A. carneus*, *A. niveus*, and *A. terreus* have all been reported to be the most important citrinin-producing fungi in nature, but other species such as *Aspergillus candidus*, have also been found to produce citrinin under laboratory conditions (Raper and Thom 1949; Frisvad and others 2006). In cheese, *P. citrinum* and *P. verrucosum* only are known to produce this mycotoxin. Citrinin appeared to be very stable in cheeses, including goat cheese, Saint Marcellin, Soignon (Bailly and others 2002). This mycotoxin is frequently associated with the presence of OTA both produced by *P. verrucosum* (Cole and Cox 1981; Ostry and others 2013).

Citrinin was shown to be nephrotoxic on all performed animal experiments (Kitchen and others 1977). LD50 is 57 mg/kg for ducks, 95 mg/kg for chickens, and 134 mg/kg for rabbits. EFSA (2012) estimated based on a 90-d toxicity study in rats, a no-observed-adverse-effect level (NOAEL) of 20 μg citrinin/kg b.w. per day for nephrotoxicity.

Citrinin can act synergistically with OTA to depress RNA synthesis in kidneys of mice (Sansing and others 1976). Citrinin also induces a loss of selective membrane permeability and cell death (possibly apoptotic) (Paterson and Lima 2010). Citrinin has a high IC_{50} *in vitro* on Caco-2 cells (83 μg/mL) but can exhibit nephrotoxic effects in mammals (Rasmussen and others 2011). Citrinin exhibits immunotoxic effects, inducing lymphocyte proliferation inhibition in pigs. An immunosuppressive effect is estimated at IC_{50} equal to 10 mg/L (Wichmann and others 2002). On embryonic stem cells, citrinin induced apoptosis via ROS and mitochondria-dependent pathway (Chan 2007).

Ochratoxin. OTA is produced by several *Aspergillus* (Benford and others 2007) and *Penicillium* species. In temperate and cold climates, *P. verrucosum* is the major producer of OTA in cereals. *P. verrucosum* is recovered mainly from plant-derived material; *P. nordicum* is generally recovered from meat and cheese products (Cabanes and others 2001). OTA has been detected in a wide range of foods and feed, including cereals, bread and beer (Visconti and others 2010) as well as meat and cheese products (Gareis and Scheuer 2000). OTA was originally discovered as an *Aspergillus ochraceus* metabolite during a large metabolite screening procedure (Van der Merwe and others 2000) and shortly these after recognized as a potent nephrotoxin, in particular in swine and poultry. Among the numerous ochratoxin analogs (at least 20), OTA is produced at the highest levels and is the most toxic. Although OTA transfer into milk has been demonstrated in other animal species, in ruminants, the resident rumen microflora decreases bioavailability through hydrolysis of OTA to OTα (Skaug 1965). Nevertheless, OTA has been detected in different commercial samples of blue-veined cheeses (Gorgonzola and Roquefort), although only at very low levels (Dall'Asta and others 1999). The same authors showed that 32.6% of blue cheeses inoculated with only *P. roqueforti* (non-OTA producers) were actually contaminated with OTA (Dall'Asta and others 2008). OTA levels detected in different cheese portions demonstrated a nonhomogeneous contamination highly associated with the blue–green veins of the cheese where fungal growth occurs. Moreover, the observed levels increased during storage, indicating that contamination was not derived from contaminated milk. This implied that OTA-producing fungal strains may have contaminated the cheese during the manufacturing process.

Ochratoxins have been known for some time to be a major etiological agent in endemic nephropathy (Radic and others 2008) and, by analogy, they have been considered to be associated with the chronic progressive kidney disease in humans known as Balkan endemic nephropathy (BEN) and urinary tract tumors (Castegnaro and others 1997; Tanchev and Dorossiev 1991; O'Brien and Dietrich 1991). Initiation of carcinogenic process is the covalent binding of OTA to DNA, forming DNA adducts (Lebrun and others 2005). OTA toxicity involves mitochondrial respiratory function, OTA interferes in modulating CYP 450 enzymes, COX, and lipox expression (Pfohl-Leszkowicz and Manderville 2006). OTA induced Ca^{2+} increase into the cells, tRNA-synthase inhibition, accompanied by reduced protein synthesis and enhanced lipid peroxidation via the generation of free radicals (Höhler 2007). Intake of OTA from cheese seems to be of limited importance for the general population in comparison to potential OTA intake from cereals and their derived products.

Mycotoxins potentially produced by the commercial cheese mold *P. roqueforti*.

Roquefortine C. Roquefortine C is the most common metabolite produced by *Penicillium* genus members (25/58 species) (Frisvad and others 1998). Moreover, according to Scott (2004) and Vinokurova and others (1981), within the *P. roqueforti* species, virtually all studied strains produce roquefortine C and its precursor roquefortine D.

Roquefortine C was previously incriminated in intoxication cases in cattle in Germany (Häggblom 2001; Auerbach and others 1990). To date, there are no toxicity studies performed on laboratory animals that meet good laboratory practice standards. Intraperitoneal (IP) lethal dose 50 (LD50) were 169 mg/kg and 184 mg/kg in male and female mice, respectively (Arnold and others 1998). Wagener and others (1987) reported paralytic activity in day-old cockerels exposed to roquefortine C. *In vitro*, roquefortine C exhibits an inhibitory concentration 50 (IC_{50}) of 48 μg/mL on Caco-2 cells (2011Rasmussen and others 1980). However, no recent data are available to estimate human exposure (Table 2). To date, roquefortine C has never shown any mutagenic activity by the Ames test (Schoch and others 1984). Roquefortine C is known to be neurotoxic (Frisvad and others 2007). There are a number of dog toxicosis cases linked to roquefortine C (Puls and Ladyman 1988), but penitrems or other compounds might have also been present.

Mycophenolic acid. Few studies reporting the presence of MPA produced by *P. roqueforti* in cheeses have been carried out on blue cheeses (Lafont and others 1979; Engel and others 1982; López-Diaz and others 1996). MPA is a phthalide and although it is a mycotoxin with potential toxicological implications, it is also a very important drug with several potential applications (Bentley 2000). It is presently used as an immunosuppressant in kidney, heart, and liver transplant patients to avoid organ rejection. Toxicity for mammals appears to be low: LD50 in rats is 2500 mg/kg (oral pathway) and 500 mg/kg by intravenous (IV) pathway in mice the LD50 is 700 mg/kg and 450 mg/kg IV (Wilson 1971). Chronic tests of 80 and 320 mg/kg daily oral doses over 1 y did not cause apparent toxicity signs in rabbits (Adams and others 1975). It is not unlikely that MPA could lower the immune system if ingested often enough thereby paving the way for bacterial infections (Bentley 2000; Frisvad and others 2007). Mutagenic activity was evaluated and showed that this mycotoxin was able to induce chromosome aberration (Umeda and others 1977) but was negative for the Ames test (Webner and others 1978).

Concerning MPA, with the exception of immunotoxicological effects, toxicological data are scarce.

Other mycotoxins. In regards to other mycotoxins produced by *P. roqueforti* in cheese, only few toxicological data are available.

Isofumigaclavines (A and B), festuclavine, and agroclavine are alkaloids potentially produced by *P. roqueforti*. They belong to the ergot alkaloid mycotoxin family (Bräse and others 2009). Isofumigaclavines A and B have been detected at low levels in commercial blue cheeses (O'Connor and O'Brien 2000). Isofumigaclavine B corresponds to the isofumigaclavine A hydrolysis product. These toxins were reported by Ohmomo and others (1975, 1977) and by Kozlovskii and others (1979). Agroclavine increased natural killer (NK) cell activity under nonstress conditions *in vivo* and *in vitro*, thus exhibiting an immunostimulatory effect (Starec and others 2001).

PR toxin (Table 2), another known mycotoxin produced by *P. roqueforti*, is unstable in the cheese environment and is converted to PR imine, an unstable and less toxic molecule. PR toxin can also be converted to PR amide in the presence of basic and neutral amino acids (Chang and others 1993). Other secondary metabolites related to PR toxin synthesis by *P. roqueforti*, such as eremofortins A, B, C, and D, have also been observed.

Mycotoxins potentially produced by the commercial cheese mold *P. camemberti*.

Cyclopiazonic acid. CPA, an indole tetramic acid, is produced by *P. cyclopium*, some *P. camemberti* strains, and several *Aspergillus* species (Pitt and others 1986). Spahr and others (2000) considered that CPA is one of the few mycotoxins that can be transferred to milk. CPA is a calcium-dependent ATPase inhibitor and induces ion transport alteration across cell membranes (Riley and Goeger 1992; Burdock and Flamm 2000). Clinical intoxication signs include weight loss, anorexia, diarrhea, dehydration, pyrexia, ataxia, immobility, and death in dogs, rats, pigs, sheep, and chickens (Bryden 1991). The possible role of CPA in aflatoxicosis cases should be explored as it could have a synergistic effect. Target organs are kidneys and gut tract in mammals. In acute toxicity, LD50 is 64 mg/kg in female mice and 12 mg/kg in chickens. In humans, CPA is suspected to be responsible for acute mycotoxicosis (named "kodua") inducing nerve troubles.

There is limited information and only few data concerning mycotoxin toxicities associated with *P. roqueforti* and *P. camemberti*. Not enough is currently known about the actual toxicity of mycotoxins found in cheeses and the toxicity of metabolites that can co-occur with mycotoxins.

Methods for qualitative and quantitative analysis (Table 1)

Due to mycotoxin toxicity, their frequent occurrence in foodstuffs, and driven by regulatory authorities worldwide, there is a distinct need for highly selective and accurate methods to identify and quantify mycotoxins in diverse agricultural and biological foods and feeds. For example, the maximum AF levels for animal feed are 50 and 20 ppb in the United States and Europe, respectively. There are no published guidelines for mycotoxin analysis. Numerous methods have been developed to analyze the large structural diversity of mycotoxins occurring in different matrices (Zöllner and Mayer-Helm 2006; Malik and others 2010).

Chromatography. Concerning cheese, classical analytical methods for mycotoxin detection and/or quantification include TLC, high-performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS) coupled with chromatography. One of the disadvantages for GC analyses for mycotoxin detection is the necessity to derivatize samples, a time-consuming and prone to error step; as a result, GC methods are less frequently used. In recent years, most of these methods have been coupled with immunoaffinity techniques to simplify extraction and improve mycotoxin recovery and measurement from foodstuffs (Bony 2000; Chu 2000; Miraglia and Brera 2000; Stroka and others 2000a, 2000b; Anon 2001; Josephs and others 2001; Krska and Josephs 2001; Turner and others 2009).

As cheese has a high fat content, hexane was simultaneously used to remove lipid compounds for the partitioning and extraction steps. However, some lipids persist during extraction; therefore, pure ACN, or methanol is also used to dissolve the sample in the final step.

HPLC is widely used to quantify different mycotoxins in cheeses, including AFM1 (Kamkar 2008). HPLC reference methods have been developed for most of the major mycotoxins and are quite sensitive with reasonably low detection levels. For example, patulin and ochratoxin are detected in traditional semihard cheeses at levels ranging from 15 to 460 and 1 to 262 μg/kg, respectively (Pattono and others 2013).

LC/MS or GC/MS have excellent detection sensitivity and selectivity. The better sensitivity of new MS instruments, such as those equipped with ion trap analyzer, in combination with better GC columns, can lead to use the GC/MS technique to significantly low patulin levels for example. However, the necessity of derivatization makes exact quantification more adapted for

LC rather than GC/MS, unless a stable isotope-labeled patulin is available as internal standard.

LC/MS is an analytical technique independent of molecular weight so that mycotoxins can be detected and quantified in complex matrices like cheeses. However, efforts must be made to develop a wide range of protocols taking into account analyte polarity diversities and different ionization capacities. Liquid chromatography tandem mass spectrometry (LC/MS/MS) is a suitable technique as it can be used to simultaneously analyze all mycotoxins produced by 1 fungus (Xu and others 2006). The HPLC/MS/MS performance for cheese samples was defined by recovery, repeatability, and limit of quantifications (LOQs). No regulatory limits exist for cheese, so LOQs cannot be evaluated.

Recently, Kokkonen and others (2005b) proposed an easy LC/MS/MS method for quantifying several mycotoxins in blue and white mold cheeses; the proposed method detected 9 mycotoxins produced by *Aspergillus* and *Penicillium* species. Mycotoxins (AFs, OTA, MPA, and roquefortin C) were quantified from cheese samples at low levels ranging from 0.6 to 5.0 μg/kg.

Liquid chromatography tandem mass spectrometry (LC/MS/MS) analytical methods are usually used to confirm data since they require expensive instrumentation and highly trained staff. Recently, the natural occurrence of masked mycotoxins (conjugated toxin) has been reported, and their detection will require even more, selective, and sensitive methods (Berthiller and others 2005).

Bioassay techniques. Bioassays have become increasingly useful for mycotoxin detection (Watson and Lindsay 1982; Yates 1986) as a rapid screening procedure before chemical analysis. Screening assays used are in majority immunochemical methods including enzyme-linked immunosorbent assays (ELISAs), lateral flow devices (LFDs), dipstick tests, fluorescence polarization immunoassay (FPIA), immunofiltration assays, and more recently, biosensor assays.

Immunochemical methods can also be considered as convenient and sensitive alternatives for detecting various mycotoxins in cheese (Chu 1991; Mak and others 2010; Mohajeri and others 2013). For example, both direct and indirect ELISA strategies have clear advantages (cost and time efficiency) and only few limitations including cross-reactivity and immunoreactivity of the primary antibody resulting in nonspecific signals (Turner and others 2009). There are a lot of commercially available bioassay kits. For example, available ELISA kits include, Agraquant (RomerLabs Diagnotica GmbH, Tulln, Austria) and Veratox (Neogen Corporation, Lesher Place Lansing, Mich., U.S.A), the LFD kit "RIDAQUICK" (R-Biopharm AG, Darmstadt, Germany) are avaible in Europe but no bioassay kit is specific to cheese samples. Additionally, not all mycotoxins (including patulin) can be measured by these methods.

As presented, numerous techniques are currently available to analyze and detect a wide spectrum of mycotoxins. However, powerful methods still need to be developed to detect and quantify multiple toxins from a single matrix. Due to the high complexity of the cheese matrix, LC/MS/MS currently seems to be the most sensitive method to detect and quantify mycotoxins in cheese.

Mycotoxin Biosynthetic Pathways

As stated above, mycotoxins correspond to a structurally diverse group varying from simple C-compounds to more complex substances (Bräse and others 2009). These compounds are part of fungal secondary metabolism, and interestingly, unlike primary metabolism-associated genes, a large number of the mycotoxin production-associated genes have been shown to be clustered on a single genetic locus (Hoffmeister and Keller 2007). This feature, combined with the fact that some of the most important mycotoxins are synthesized by polyketide synthases (PKSs) (AFs, fumonisins, ochratoxins, and zearalenone), nonribosomal peptide synthetases (NRPS) (gliotoxin, enniatin, and HC-toxin), or PKS-NRPS hybrid proteins (CPA), allowed to identify biosynthetic pathways and the involved genes in various species (Figure 1).

Biosynthetic pathways in undesirable species

For cheese-contaminating fungi, the pathways associated with mycotoxin production have been studied in depth from a genetic point of view.

Aflatoxins/sterigmatocystin. The biosynthetic genes and regulation involved in AF and STC synthesis have been extensively documented in *Aspergillus* species (Keller and Hohn 1997; Minto and Townsend 1997; Payne and Brown 1998; Brown and others 1999; Yu and others 2004; Yabe and Nakajima 2004; Georgianna and Payne 2009; Huffman and others 2010) including *A. flavus* (pathway discovered in this AF producing cheese contaminant) (Barrios and others 1998; Mogensen and others 2010) and *A. parasiticus* (Yu and others 2004). In regards to cheese contamination and as previously stated, detection of these mycotoxins in cheeses is typically due to indirect cheese contamination via feedstuffs (this is the case for AFs and AFM1 produced by *Aspergillus* species on feedstuffs that are further detected in milk and cheese) or postcontamination (mostly relates to STC in cheese due to *A. versicolor* mold growth on cheese surfaces (Northolt and others 1980). Among the studied chesse-related and noncheese-related species, gene organization can differ and may indicate different evolutionary histories; this is the case of *A. nidulans* and *A. flavus* (Cary and others 2009). For cheese-related *Aspergillus* spp., the AF biosynthetic pathway was shown to contain 25 genes clustered within a 70-kb region and involves at least 23 enzymatic reactions (Yu and others 2004; Huffman and others 2010). The starter units correspond to acetate and malonyl-CoA that are converted sequentially to hexanoate by a fatty acid synthase (FAS) and to norsolorinic acid (common to both AF and STC biosynthesis) by a PKS (Crawford and others 2006, 2008a, 2008b, 2009). Expression of all pathway genes, except for aflS (formerly known as aflJ and also involved in AF regulation), has been shown to be positively regulated by a transcription factor, aflR. It is known that STC is the final metabolic product of the biosynthetic pathway in *A. nidulans*, while this mycotoxin acts as a precurseur for AF biosynthesis in other *Aspergillus* species including *A. flavus* and *A. parasiticus* (Yu and others 2004; Huffman and others 2010). These biosynthetic pathways have been extensively reviewed by Yu and others (2004) and Huffman and others (2010), and the detailed gene cluster is provided in Figure 1 for *A. flavus*, a species involved in indirect mycotoxin contamination in milk and cheese.

Ochratoxin. A pathway for OTA biosynthesis was recently reviewed by Huffman and others (2010) and includes a polyketide synthase for PK dihydroisocoumarin synthesis, a methyltransferase, a P450-type oxidation enzyme for carboxyl group formation at C7, a NRPS to catalyze ligation between phenylalanine and PK and a halogenase to incorporate the chlorine atom. To date, characterization of part of the OTA biosynthetic gene cluster has been described in the cheese contaminating species *Penicillium nordicum* (Karolewiez and Geisen 2005; Geisen and others 2006). Geisen and others (2006) sequenced a 10-kb-length fragment containing 3 genes corresponding to a partial PKS (otapksPN), a complete NRPS homolog (npsPN) and a complete alkaline serine protease homolog (aspPN). Moreover, O'Callaghan and others

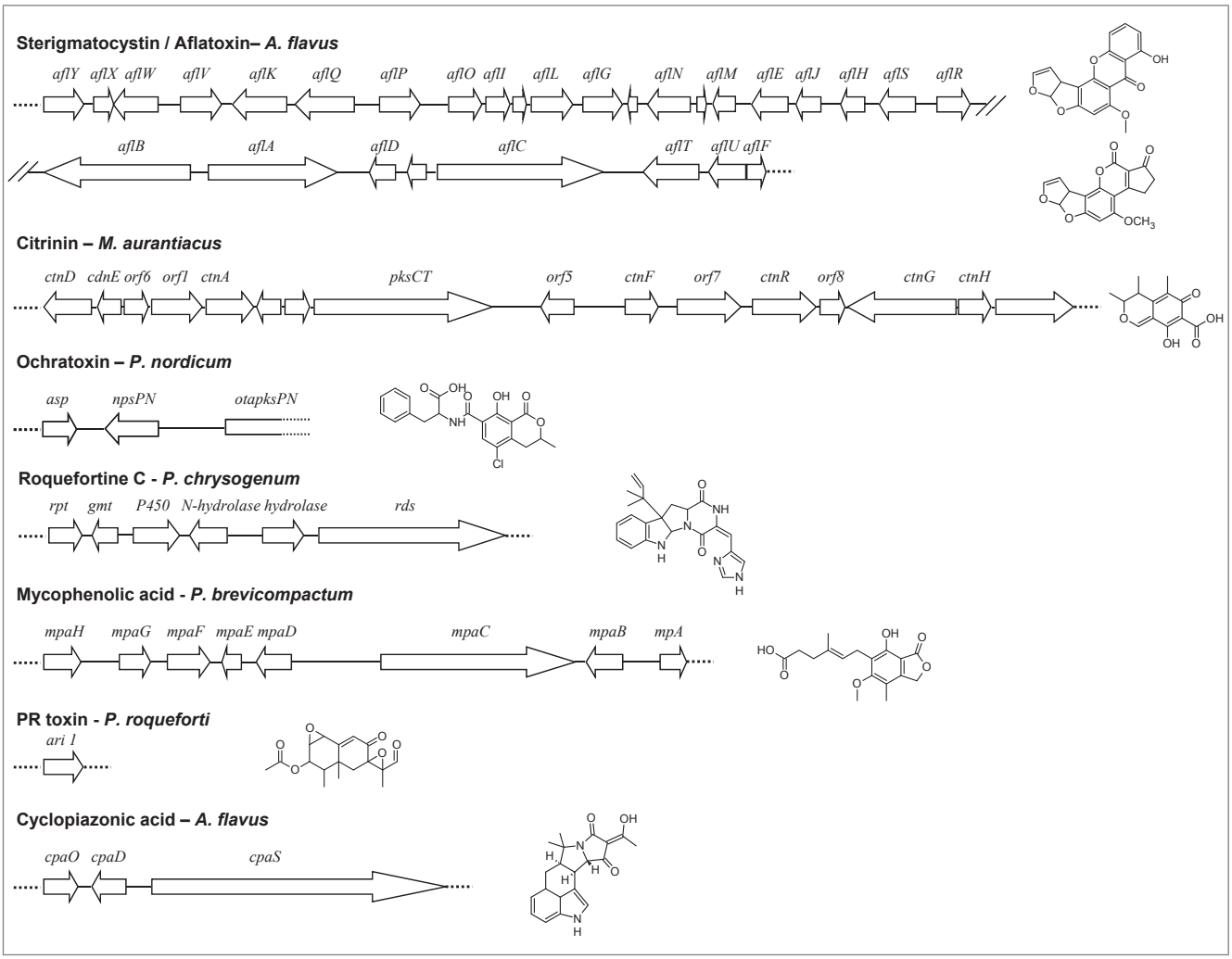

Figure 1–Known fungal gene clusters associated with production of mycotoxins encountered in cheese. Based on the following Genebank accession numbers: sterigmatocystin/aflatoxin (NW_002477243), citrinin (AB243687), ochratoxin (AY557343), roquefortine C (AM920436), mycophenolic acid (HQ731031), PR toxin (L05193), and cyclopiazonic acid (JN712210).

(2013) recently described a 3-gene cluster encoding an oxidoreductase (OtaE), a PKS (OtapksPV), and a transporter protein (OtaT) involved in OTA biosynthesis in *P. verrucosum*, another cheese contaminating species. Other *Penicillium* species carry inactive homologs for these genes (*P. nalgiovense*, also isolated from cheese) or do not carry these genes. However, detailed genetic and biochemical studies are still required to elucidate the molecular mechanisms and confirm the proposed OTA biosynthetic pathway.

Citrinin. Citrinin is known to be produced by some cheese contaminating *Penicillium* species including *P. citrinum*, *P. verrucosum*, and *P. expansum* (Ei-Banna and others 1987; Kurata 1990; Cabanes and others 2007). However, to this date, citrinin biosynthetic pathway has not been characterized in these species but can be extrapolated from putative citrinin biosynthetic 16-gene cluster recently described in *Monascus purpureus* (Shimizu and others 2005, 2007) and *M. aurantiacus* (Li and others 2012). In these species, the pathway genes were identified to be part of a putative 43-kb biosynthetic gene cluster including a polyketide synthase gene (pksCT), a transcriptional activator gene (ctnA), a membrane transporter gene (orf5), a fatty acyl-CoA synthetase (ctnI), an oxygenase (orf3), 3 genes for post-PKS-modifying enzymes, oxidoreductase genes (ctnD, orf4), and 3 dehydrogenases (ctnE, orf1, ctnH) among others (Shimizu and others 2005; Li and others 2012). Biosynthesis of citrinin, originating from a polyketide, often co-occurs with OTA. Further studies are still required to fully understand the regulation of citrinin biosynthesis.

Biosynthetic pathways in cheese ripening species

Concerning the potentially mycotoxigenic fungal species used as ripening cultures for cheese, *P. roqueforti* and *P. camemberti*, biosynthethic pathway-associated genes have not been described to this date, with the exception of the PKS involved in PR toxin formation. However, pathways have been identified in other mold species. These studies can be used as a base for studying mycotoxin-related genes in fungi used as ripening cultures in cheesemaking.

Roquefortine C. Studies have shown that roquefortine C is an intermediate of the oxaline and epi-neoxaline pathway in *P. glandicola* (Reshetilova and others 1995) and *P. tulipae* (Overy and others 2005), respectively. In 2011, a biosynthesis pathway was proposed in *P. chrysogenum* (Garciá-Estrada and others 2011). In this study, by using gene silencing, the authors indicated that a single-gene cluster containing 6 genes involved in the biosynthesis and secretion of 2 mycotoxins: roquefortine C and meleagrin.

The cluster organization showed the presence of genes encoding a nonribosomal cyclodipeptide synthetase, a prenyltransferase, and an N-hydroxylase leading from histidine and tryptophan to roquefortine D. Under the action of a monooxygenase, the latter substance is metabolized into roquefortine C. The production of meleagrin is obtained *via* the actions of 3 enzymes leading sequentially to glandicoline A and glandicoline B before meleagrin. More recently, 2 publications refined the biosynthetic pathway and reassigned ascribed synthetases. According to Hazrat and others (2013), the NRPS RoqA is indeed responsible for the production of histidinyltryptophanyldiketopiperazine (HTD). The molecule can be transformed by the sequential action of RoqR followed by RoqD, or conversely by the action of RoqR and then RoqD, to lead to roquefortine C. This is done without the action of RoqM contrary to what was proposed by Garciá-Estrada and others (2011). The same research group showed further branching in the metabolic pathway by the characterization of roquefortine F and neoxaline as well as the identification of Roquefortine L and its degradation products (Ries and others 2013). Noteworthy, in *P. roqueforti* and in the cheese context, only roquefortine C has been observed, thus suggesting that a part of the pathway leading from roquefortin C to meleagrin might either be absent or inactive in this species.

Mycophenolic acid. As described by Birch and others (1957), MPA consists of an acetate-derived phthalide nucleus and a terpene-derived side chain indicating the involvement of polyketide and isoprenoid pathways. Proposed biosynthetic pathways (Bentley 2000; Regueira and others 2011) indicate that acetyl-CoA, malonyl-CoA (x3), and S-adenosyl methionine (SAM) would form 5-methylorsellinic acid (5-MOA) through the action of a polyketide synthase. Following phthalide formation, a reaction with farnesyl diphosphate yields 6-farnesyl-5, 7-dihydroxy-4-methylphthalide. Degradation of the side chain via oxidative cleavage leads to demethylmycophenolic acid (DMPA), which is finally methylated by a SAM: DMPA O-methyltransferase to yield MPA. This last step was confirmed by the purification and characterization of SAM: DMPA O-methyltransferase in *P. stoloniferum*. Regueira and others (2011) identified and characterized the MPA biosynthetic gene cluster (8 putative genes) in *P. brevicompactum*. Gene cluster determination was performed by detection of a resistance gene encoding an inosinate dehydrogenase (IMPDH) and deletion of the mpaC gene encoding a PKS, which abolished the production of metabolites (including MPA), associated with MPA biosynthesis. In the described cluster, the mpaG gene encodes the SAM: DMPA O-methyltransferase. Recently, Hansen and others (2012) showed through the creation of a chimeric MpaDE protein, consisting of a cytochrome P450 (encoded by the mpaD gene) and a hydrolase (encoded by the mpaE gene), that the step following 5-MOA production in the pathway could be carried out by a natural-fusion MpaDE enzyme.

PR toxin. PR toxin metabolic pathways have been proposed in *P. roqueforti* (Moreau and others 1980; Chalmers and others 1981; Jelén 2002). In the pathway, Chalmers and others (1981) proposed that the pathway precursor, acetate, is sequentially converted to mevalonate, dimethylallyl pyrophosphate (DMAPP), and farnesyl pyrophosphate (FPP). Cyclizations by the aristolochene synthase lead to aristolochene, the precursor for eremophilane-type sesquiterpenes, with germacrene A as an intermediate, as demonstrated by Calvert and others (2002). PR toxin arises from aristolochene *via* a synthetic pathway including eremefortin B, A, and C. Eremefortin C is finally converted to PR toxin. The acetal form of eremofortin C leads to eremofortin D (Moreau and others 1980). Recently, Brock and Dickschat (2013) used a combination of CLSA/GC-MS and ^{13}C NMR to obtain an in-depth view of the biochemical pathway and described several unknown sesquiterpenes leading from FPP and aristolochene as well as side products. PR toxin can be degraded in PR imide, PR amide (eremofortin E) (Chang and others 1993), and PR acid (Chang and others 1996). Concerning the genes and enzymes associated with this pathway, until recently, the genes involved in this pathway had not been described, with the exception of aristolochene synthase, a sesquiterpene cyclase, implicated in PR toxin biosynthesis in *P. roqueforti* (Proctor and Hohn 1993). The 1129-bp-long Ari1 gene, exhibiting 2 introns, encodes a 342-amino acid protein with a calculated molecular weight of 39200 (Proctor and Hohn 1993). The protein was purified by Hohn and Plattner (1989) and was shown to be Mg^{2+}-dependent. The crystal structure (2.5-Å resolution) was determined by Caruthers and others (2000) and confirmed that the enzyme is responsible for the cyclization of the FPP, to form aristolochene. The enzyme eremefortine C, responsible for the conversion of eremefortin C to PR toxin, was studied by Chang and others (1985). Recently, Hidalgo and others (2014) cloned and sequenced a 4-gene cluster from *P. roqueforti*. The cluster included the *ari1* gene and silencing of the other genes (encoding for putative oxidoreductases and an alcohol dehydrogenase) demonstrated their involvement in the PR synthetic pathway. Interestingly, silencing of the PR toxin production caused a large increase in MPA synthesis suggesting a cross talk between these 2 mycotoxin pathways.

Cyclopiazonic acid. CPA, produced by *P. camemberti* in cheese, derives from tryptophan, acetyl CoA, malonyl CoA, and DMAPP, as a prenyl donor, via the formation of 2 intermediates, cycloacetoacetyl L-tryptophan (cAATrp) and β-CPA (Holzapfel and Wilkins 1971). As stated above and as for the other mycotoxins described in this section, to date no complete biosynthetic cluster associated to cheese ripening cultures has been elucidated. However, for CPA, gene clusters were described in *A. flavus* (cheese-contaminating species) and *A. oryzae* and can be used as a base to elucidate the corresponding pathway in *P. camemberti*. In *A. flavus*, the CPA biosynthetic pathway consisted of 3 genes (cpaS, cpaD, and cpaO) and was situated next to the AF biosynthesis gene cluster (Chang and others 2009b). In *A. oryzae*, the ability to form CPA is strain-dependent (Chang and others 2009a); while the cluster is complete in the NBRC 4177 strain, the RIB40 strain is unable to form the mycotoxin due to a truncation of the PKS–NRPS (Shinohara and others 2011). Studies performed by Liu and Walsh (2009a, 2009b) demonstrated that cpaS encodes a PKS–NRPS responsible for cAATrp formation, while cpaD encodes a cAATrp-dimethylallyltransferase, leading to β-CPA. In this cluster, the cpaO gene encoding a monoamine oxidase would lead to CPA, thus correlating well with the previously proposed CPA biosynthetic pathway. The study by Chang and others (2009b) showed that disruption of this gene (named maoA in the study) abolished the production of CPA in *A. flavus*.

Concerning mycotoxin biosynthetic pathways, the already described gene clusters as well as the growing access to complete fungal genomes will allow identifying the corresponding pathways in cheese-related fungi. Moreover, the presence of mycotoxin-related genes in the subphylum *Mucoromycotina*, so far considered as a nonmycotoxin-producing group, could be evaluated.

Factors Modulating Mycotoxin Biosynthesis in Cheese

It is generally accepted that fungal growth and mycotoxin production in food is influenced by multiple factors including abiotic

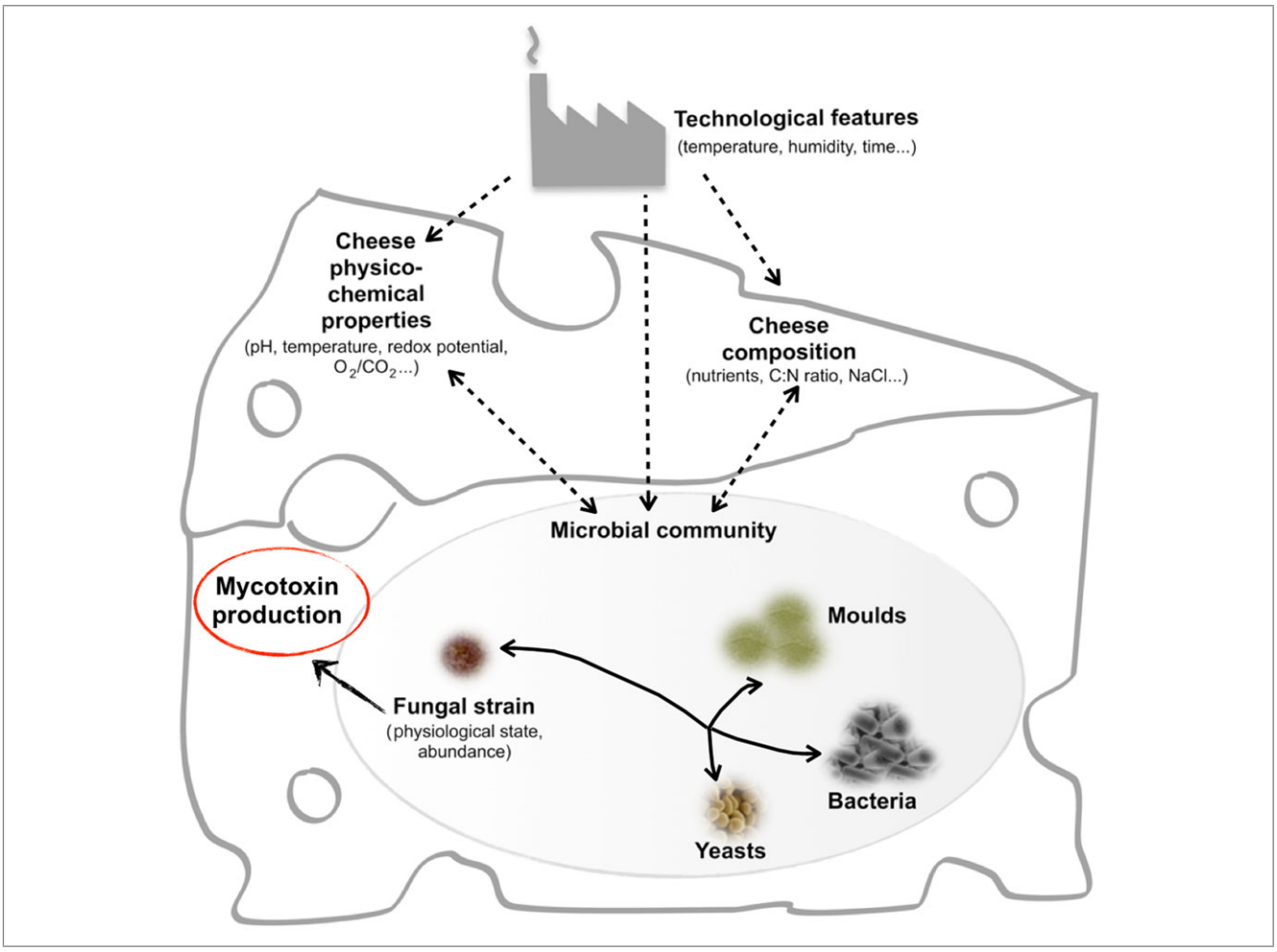

Figure 2–Main biotic and abiotic factors modulating mycotoxin production in cheese.

and biotic factors (Filtenborg and others 1996). In cheese, biotic factors modulating mycotoxin production include the intrinsic capacity of a given mold species or strain to produce mycotoxins, its physiological state and the interactions that may occur between this organism and the other members of the cheese microbiota (Figure 2). The main abiotic factors that are likely to modulate mycotoxin production in cheese can be divided into manufacturing and environmental factors such as temperature, relative humidity (RH), ripening time, and storage atmosphere used during cheesemaking, as well as physicochemical factors of the cheese itself, including chemical composition (carbon and nitrogen sources, C/N ratio, NaCl content . . .), pH, water activity (a_w), and redox potential ($E°$) (Figure 2). Because over 1000 cheese varieties exist worldwide, it is difficult to draw a general picture of abiotic factor variations in cheeses. For example, in surface-ripened cheeses comprising soft, semihard, and hard types, cheeses are ripened at an RH ranging from 90% to 80% to 85% and at temperatures of 4 to 20 °C for 14 to 540 d depending on cheese type (Robinson 1995). Moreover, pH, NaCl, and a_w at the cheese surface layer may vary at the end of ripening from 7% to 5.6%, 2% to 0.6%, and 0.98% to 0.95%, respectively (Robinson 1995).

The physical and chemical characteristics of cheeses are also subjected to both spatial and temporal changes during ripening. First, environmental conditions used in cheese manufacture, such as RH and temperature in ripening rooms and biological activities (glycolysis, proteolysis, and lipolysis) of cheese microorganisms, affect physicochemical cheese characteristics during ripening. Moreover, cheese is also characterized by a heterogeneous physicochemical composition with gradients in pH, NaCl, moisture, a_w, O_2 level, and so on from the surface to the core. For example, in the core and surface of the blue-veined cheese Danablu, pH, NaCl, and a_w gradients ranged from 4.6% to 4.4%, 1% to 6.0% and 0.99% to 0.87%, respectively, after 1 week of ripening while they ranged from 6.4% to 5%, 2% to 4.5% and 0.94% to 0.91%, respectively, after 5 wk of ripening (Cantor and others 2004). Also in Danablu cheese, van den Tempel and others (2002) indicated that average oxygen saturation decreased from 63% to 0.1% at the center and from 69% to 0.1% at the cheese edges from 1 to 19 wk. It is also interesting to note that in the same study, after 3 wk of ripening, oxygen was not detected in the cheese, except in the 0.25 mm surface layer and in apparent small air pockets within the cheese containing about 3% oxygen.

Effect of nutritional factors on mycotoxin production

While it is generally admitted that the chemical composition of food exerts a significant effect on mycotoxin production, there is controversy in the literature on whether cheese is a food matrix that is well suited for mycotoxin production. In earlier and much more recent studies, cheese was considered a poor substrate for mycotoxin formation because of its low C/N ratio (Scott and

others 1977; Engel 1978; Olivigni and Bullerman 1978; Finoli and others 2001) and low ripening temperature (Marth 1979). Finoli and others (2001) investigated the ability of *P. roqueforti* fungal ripening cultures to synthesize roquefortine C and PR toxin *in vitro* in 10% reconstituted skim milk and yeast extract sucrose (YES) medium, which is characterized by a high C/N ratio due to its high concentration of sucrose (150 g/L). They also analyzed, using a HPLC-UV method, roquefortine C and PR toxin contents in 30 blue-veined cheeses from where the strains originated. The cheeses contained very small amounts of roquefortine C (<1.44 mg/kg) and no PR toxin was detected. Roquefortine C and PR toxin were generated in very low quantities (<3.08 mg/L), if any, in milk, whereas all the studied strains produced these 2 mycotoxins in YES medium with up to 8.44 and 60 mg/L for roquefortine C and PR toxin, respectively. In contrast, Kokkonen and others (2005a), using LC/MS/MS, showed that roquefortine C was present in 11 blue mold cheese samples purchased from Finnish supermarkets at concentrations ranging from 0.8 to 12 mg/kg that were about 10 times higher than those reported by Finoli and others (2001). However, it is difficult to compare the concentrations of roquefortine C reported in the latter 2 studies because different methods were used for quantification. More importantly, different cheese varieties likely obtained with different strains of *P. roqueforti* and ripening conditions were analyzed. More studies on a large number of blue cheese samples and varieties are therefore needed to clarify whether or not cheese is well suited for roquefortine C production and the relationships existing between roquefortine C content, manufacturing practices and the toxigenic potential of *P. roqueforti* strains. This observation is also true for other mycotoxins such as CPA and MPA.

Kokkonen and others (2005b) also studied the ability of *P. crustosum*, *P. nordicum*, and *P. verrucosum* to produce mycotoxins in 3 different substrates: YES agar as well as cheese and bread analogs. *P. nordicum*, a frequent contaminant encountered in cheese, only synthesized moderate or undetectable amount of OTA and none of the substrates favored its production. In contrast, the substrates had a significant impact on the secondary metabolism of the other tested fungi. *P. crustosum* produced roquefortine C on all the substrates, with the highest amount on the cheese analog, while it only synthesized penitrem A on the cheese analog that was a favorable medium for the production of this mycotoxin. The authors hypothesized that the ability of *P. crustosum* to produce this secondary metabolite was linked with the high protein content of cheese because amino acids are required for the synthesis of tremorgenic mycotoxins. In contrast, only 1 out of 8 *P. verrucosum* strains produced OTA on the cheese analog, while high levels of ochatoxin A and citrinin were produced on the bread analog. Interestingly, fresh goat cheese was also a more favorable substrate for citrinin biosynthesis by *P. citrinum* and *P. expansum* than YES medium with up to 600 mg citrinin per kg of cheese produced after 10 d of incubation at 20 °C (Bailly and others 2002). However, as discussed by Kokkonen and others (2005b), the presence of precursors in the substrate cannot solely explain the ability of a given species/strain to produce higher levels of mycotoxins, and the factors and their interrelations that modulate mycotoxin production in food are mostly unclear. For example, in the study of Finoli and others (2001), roquefortine C production in cheese or milk by *P. roqueforti* was poor, despite the fact that the cheese contained high protein levels including the mycotoxin precursors (tryptophan and histidine).

In conclusion, it cannot be assumed that cheese is a poor substrate in terms of its nutritional composition for mycotoxin production. Most probably, the ability of a mold to produce mycotoxins in cheese is rather species-or strain-specific. Moreover, it also appears that the nature and the quantity of mycotoxins produced by a given mold strain are influenced by the substrate composition as well as some other intrinsic or environmental factors discussed below.

Effect of NaCl on mycotoxin production

Sodium chloride in cheese contributes to flavor and acts as a preservative through water activity reduction and microorganism inhibition due to solubilized ions. It also enables the growth of salt-tolerant microorganisms that mostly contribute to the sensory properties of cheese. However, NaCl may also affect mycotoxin production. Indeed, Schmidt-Heydt and others (2012) showed that high amounts of OTA were produced by *P. nordicum* over a wide concentration range of NaCl (5 to 100 g/L), with a weak optimum at 20 g/L in YES medium. A mutant strain of *P. nordicum*, unable to produce OTA, displayed important growth reduction and elevated chloride content in mycelium under elevated NaCl concentration. It was also shown that *P. verrucosum* shifted from production of citrinin to ochratoxin when NaCl concentration was equal to or above 20 g/L with a peak at 40 g/L NaCl. As presented above for the possible roles of mycotoxin production in molds, these observations suggest that the biosynthesis and excretion of OTA, containing a chloride atom, plays a role in maintaining chloride homeostasis in the fungal cell.

Finoli and others (2001) observed that there was a positive relation ($r^2 = 0.85$) between the amount of roquefortine C and NaCl concentration in various blue cheeses. However, due to the number of varying intrinsic factors, it could not be extrapolated from the latter study whether NaCl alone had an effect on mycotoxin production by *P. roqueforti*. For example, Finoli and others (2001) also found a positive relation ($r^2 = 0.58$) between ripening time and roquefortine C concentration. Further work is therefore needed to understand how the range of NaCl concentrations commonly encountered in cheeses affect production of mycotoxins other than citrinin and OTA.

Effects of temperature, a_w, pH, and atmosphere composition on mycotoxin production

Whatever the fungal species (contaminant or fungal ripening cultures), observations have been made, both *in vitro* and in cheese, that mycotoxin-producing fungi are inversely correlated with temperature (low or no production at refrigerated temperatures, intermediate production at 12 °C, and optimal production at 20 to 25 °C) (Le Bars 1979; Wagener and others 1980; Chang and others 1991; Sweeney and Dobson 1998; Finoli and others 2001; Taniwaki and others 2001; Bailly and others 2002; Erdogan and others 2003). This is not surprising, since low temperature also affects fungal growth that is often, but not systematically, correlated with mycotoxin production.

Very little information is available on the effects of a_w and pH on mycotoxin production by cheese-related fungi (ripening cultures and common contaminants, see "Filamentous Fungi in Cheeses: The Good and the Bad" section). Most fungi able to grow in cheese are salt-tolerant and can grow at relatively low a_w conditions. In *P. verrucosum*, OTA production in YES medium was enhanced at both near-optimal and under mild pH and a_w stress conditions (Schmidt and others 2008). However, this does not mean that mycotoxin production did not occur with other conditions since production also occurred at other pH and a_w values (Schmidt and others 2008). In *P. roqueforti*, the optimum pH for

eremofortin C and PR toxin production was around 4.0 in modified YES medium (Chang and others 1991). However, no information is currently available on the effect of a_w on the production of these secondary metabolites or other secondary metabolites such as roquefortine C. More studies are also needed regarding the influence of these factors on mycotoxin production by the most common ripening cultures (*P. camemberti, P. roqueforti, F. domesticum, S. flava, and S. fusca*) and contaminants (*P. commune*, *P. palitans*, *P. nalgiovense*, and *P. nordicum*) mentioned in "Filamentous Fungi in Cheeses: The Good and the Bad" section.

Gas composition (CO_2 and O_2 levels) of the atmosphere may also exert a significant impact on mycotoxin production by cheese-related fungi (Taniwaki and others 2001, 2009, 2010). Modified atmosphere packaging (MAP), which combines the inhibitory effect of low oxygen and elevated carbon dioxide levels, is increasingly used as a hurdle technology to prevent spoilage fungi to grow in cheese (Haasum and Nielsen 1998). Taniwaki and others (2001) compared mycotoxin production by strains of *A. flavus*, *P. roqueforti*, and *P. commune* on sliced-Cheddar cheese in air and the combined presence of CO_2 (20% or 40%) and O_2 (1% or 5%). Mycotoxin production was greatly decreased under modified atmosphere conditions compared to production in air. For example, roquefortine C and CPA production were divided by factors of 7- to 20-fold and 12- to 850-fold under modified atmospheres as compared to mycotoxin production in air by *P. roqueforti* and *P. commune*, respectively. On the other hand, the gas composition in the cheese core may also influence secondary metabolite production by *P. roqueforti* during blue-cheese ripening. In blue-veined cheeses such as Danablu cheese, O_2 levels decrease rapidly during the 1st weeks of ripening, while the highest CO_2 level reported in Danablu cheese was 20% (van den Tempel and Nielsen 2000). Consequently, these atmosphere conditions are most likely unfavorable for mycotoxin production in cheese.

Effects of biotic factors

To our knowledge, the intraspecific mycotoxigenic potential of fungal species found in cheese has not yet been extensively studied. Nevertheless, the toxigenic potential of different strains of *P. roqueforti* and *P. camemberti* was highly variable (Le Bars 1979; Chang and others 1991; Finoli and others 2001). As already mentioned, the quantity and nature of secondary metabolites produced may be a strain-dependent trait. For example, high intraspecific differences in MPA/PR toxin ratios have been reported in *P. roqueforti* (O'Brien and others 2006).

Microbial interactions play an important role in cheese quality and safety (Irlinger and Mounier 2009). Cheese microbiota or the addition of protective cultures may provide a hurdle effect (competitive interactions, production of inhibitory compounds) toward potentially toxigenic fungal contaminants that, in turn, are unable to produce mycotoxins (Nielsen and others 1998; Irlinger and Mounier 2009; Dalié and others 2010). Finally, it is worth noting that very little attention has been given to studying the effects of cheese cultures with beneficial use on mycotoxin production by fungal ripening cultures.

As mentioned above, further work is still required to fully understand the effect of biotic and abiotic factors on fungal growth and subsequent mycotoxin production in cheese. The effects of single abiotic factors and their interactions on mycotoxin formation and the intraspecific variability within mycotoxigenic spoilage fungi and fungal ripening cultures deserve further attention. With the development of mathematical models able to predict fungal growth and mycotoxin production, the cheese industry will be able to apply them to both existing and newly developed products. This will allow companies to better define manufacturing processes, storage conditions, shelf-life, and will contribute to the foodborne mycotoxin risk assessment (Membré and Lambert 2008; Garcia and others 2009).

Control of Mycotoxins in Cheeses

As previously stated, some authors consider that cheese is very susceptible to mold growth as well as mycotoxin production (Sengun and others 2008). On the contrary, other authors (Gourama 1997) have indicated that conflicting reports can be found in the literature concerning mycotoxin production in cheese and dairy products, and concluded that cheese is actually a better medium for mold growth than for mycotoxin production. The impact of toxic molds on cheese is relatively low and nontoxic molds overgrow toxic ones; moreover, cheese is a poor substrate for mycotoxin production if correctly stored at a low temperature (5 to 7 °C) (Bullerman 1981). However, even if the risk is considered to be low, it should be controlled. The 1st goal is to avoid mycotoxins in milk. Then, during cheese production and storage until consumption, 2 different aspects should be considered, fungal contaminants that potentially produce mycotoxins are undesirable and should be avoided and fungal ripening cultures intentionally added during cheesemaking (for example, blue-veined cheeses or camembert-type cheeses) should not be mycotoxigenic.

Control of mycotoxins in milk

Good animal feeding practices are necessary to avoid mycotoxins in feed products and subsequently, in milk (mainly AFM1). Molds able to produce mycotoxins may grow on plant materials in the fields before harvest, during handling and storage, as well as during transformation into feed products (Jouany 2007).

During harvest and postharvest steps, mycotoxin control includes: early harvest usually associated with low concentrations of mycotoxins, high cutting heights to avoid contamination from soil particles and elimination of damaged grains that can favor fungal growth, low humidity during storage (the main factor to avoid fungal growth, critical limit of 14% w/w), and cool storage temperatures and ventilation that are important secondary factors to reduce the growth risk (Jouany 2007).

For silage, humidity remains relatively high; therefore, is it necessary to respect the optimal moisture content at harvest (33% to 35% dry matter for the entire plant [Demarquilly 1994] and use good agricultural practices when filling silos). By following these guidelines, anaerobic conditions are encountered and lactic fermentation takes place thus avoiding fungal growth.

LAB, known for their antifungal activities, can be inoculated during silage production and are now more frequently used than chemical preservatives (Kalac 2011). A *Lactobacillus casei* subsp. *pseudoplantarum* 371 isolate from silage, was studied for its ability to inhibit mold growth and *A. flavus* subsp. *parasiticus* NRRL 2999 AF production (Gourama and Bullerman 1997). Numerous biological additives, usually containing LAB, are currently marketed for silage control. However, the main recommendations for usual practice are to quickly create stable anaerobic conditions and to apply propionic acid, well known for its antimold activity (Kalac 2011). In Europe, propionic acid (E code = E280) is authorized for use in feed without a time limit and, consequently, different acidic additives for silage containing propionic acid are currently sold.

Concerning milk containing mycotoxins, curative physical, chemical, and/or biological treatments to eliminate mycotoxins

are poorly efficient and too expensive to bring viable solutions for feed decontamination (Jouany 2007; Kolossova and others 2009). Different physical and chemical methods have been recommended to detoxify food and feed; however, only a few (ammonia treatment to destroy AF for instance) are practically used (Bata and Lásztity 1999). Up to 95% to 98% of AF is decomposed by ammoniation that is consequently used in various countries for animal feedstuffs decontamination (van Egmond 2004).

Milk-producing animals are more or less efficient barriers against mycotoxins. Rumen microorganisms and the liver produce toxic metabolites (Kalac 2011). However, of healthy animal rumen microbial communities also contribute to prevent health risks as they biotransform different mycotoxins (for instance, deoxynivalenol or zearalenone, the main mycotoxins produced in silage) into harmless metabolites. Consequently, milk usually contains low concentrations of mycotoxins.

Mycotoxins are generally stable compounds. However, heat treatment and ultraviolet light application have been shown to be efficient methods to reduce AFM1 content in milk (Rustom 1997).

Control of mycotoxigenic fungal contaminants in cheese

Good hygiene and sanitation standards throughout the entire food chain permit to minimize mold presence and growth on cheese (Bullerman 1981).

Cold storage. Cold storage (5 to 7 °C) is an important step in mycotoxin prevention (Sengun and others 2008). *Penicillium* species are the only molds growing at such temperatures (Bullerman 1981) and are less problematic as they do not produce AFs or STC. Such species could produce less toxic and less stable metabolites in cheese (such as MPA). At the consumer level, cheeses should not be maintained at room temperatures unless other prevention techniques are employed.

Chemical preservatives. Chemical preservatives can be used to control mycotoxins (Sengun and others 2008). For instance, pimaricin (= natamycin, E code = E235, produced by the actinobacterium *Streptomyces natalensis*) was shown to delay fungal growth and as a consequence to avoid mycotoxins (Kiermeier and Zierer 1975; Nilson and others 1975). Its direct effect on production of mycotoxins in cheese remains poorly documented. This antifungal agent is now permitted and used in cheese in Europe and the United States. Private companies sell natamycin formulations for use in cheese production. In Europe, the admissible daily intake value is 0.3 mg/kg of body weight. Mold growth was inhibited in vacuum-packed Kashar cheese containing natamycin, for a 5-mo ripening period at 4 °C (Var and others 2006).

Preservatives such as sorbic, benzoic, and propionic acids can inhibit mold growth, except for *P. roqueforti*. This species is resistant and, consequently, the most important spoilage species, except for blue-veined cheeses (Filtenborg and others 1996). The U.S. Federal Standard of Identity permits the use of sorbate in cheeses at levels under 0.3%, calculated as sorbic acid (Sengun and others 2008). Propionates are permitted for the surface treatment (such as wrapping materials) of cheese and cheese products (Nielsen and de Boer 2004).

Mold growth on cheeses can also be reduced using high sodium chloride concentrations that decrease water activity required for growth and mycotoxin production (Sengun and others 2008); however, both organoleptic factors and health considerations have to be taken into account in this context.

Reduction or absence of oxygen. Since mycotoxin-producing molds are obligate aerobes, mold growth and mycotoxin formation are minimized by low oxygen concentrations and/or high concentrations of other gases (CO_2) in the gaseous phase (Bullerman 1981). Consequently, vacuum-packaging is used to inhibit fungal growth, and in such conditions, production of roquefortine C and CPA by *P. roqueforti* and *P. commune* is virtually eliminated (Taniwaki and others 2001).

Plant extracts. Knowing their organoleptic characteristics, antimicrobial herbs, spices, or their essential oils have long been used to prevent fungal growth in cheese (Sengun and others 2008). On this basis, eugenol and thymol were shown to affect growth and citrinin production by 2 *P. citrinum* strains in Spanish cheeses (Vázquez and others 2001). Eugenol appeared more efficient than thymol and the effects reported were cheese-dependent. Fungal growth was totally inhibited in Arzu´a-Ulloa cheese with 200 mg/mL of eugenol, but no effect was observed in Cebreiro cheese. At lower concentrations, permitting fungal growth, citrinin production inhibition appeared to be limited or even absent.

Microbial control. Competing and/or inhibiting microorganisms can also limit fungal growth and mycotoxin production (Sengun and others 2008). When fully viable, the secondary ripening cultures *P. camemberti* and *G. candidum* can efficiently compete against contaminants (Nielsen and others 1998; Decker and Nielsen 2005). Species belonging to the *Lactococcus* and *Lactobacillus* genera are the most capable of preventing or limiting mycotoxigenic mold growth because of organic acid production, competition for nutrients, and the production of other antagonistic compounds (Dalié and others 2010). Raw milk from ewe, cow, and goat was shown to be a productive reservoir for antifungal bacteria, most of them belonging to *Lactobacillus* (Delavenne and others 2011). Eleven antifungal *Lactobacillus* species showed an antifungal activity in milk with strain-dependent activity spectra (fungal species tested were *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Penicillium brevicompactum*, *Rhodotorula mucilaginosa,* and *Yarrowia lipolytica*). *Lb. harbinensis* showed very strong antifungal effect in yogurt inhibiting all tested fungi. Lactobacillus amylovorus DSM 19280 was shown to inhibit *Penicillium expansum, P. roqueforti, Aspergillus niger, Aspergillus fumigatus*, and *Fusarium culmorum*. In the presence of the bacterial adjunct, cheddar cheeses exposed to natural airborne fungi benefited from a 6-d delay in the appearance of mycelia on their surface without detectable negative impact on cheese quality (Lynch and others 2014).

Dairy propionibacteria, widely used for Swiss-type cheese production, are known to ferment glucose, lactic acid, and glycerol into propionic acid, acetic acid, and carbon dioxide. Antifungal effect of dairy *propionibacteria* was studied using glycerol as carbon source for bacterial growth. Five type strains of *propionibacteria* were tested against the yeast *Rhodotorula mucilaginosa* and the molds *Penicillium commune* and *Penicillium roqueforti*. Increase in glycerol concentrations enhanced the inhibition of the molds when the yeast was less affected. This effect was shown to be due to the production of propionic acid and simultaneous pH reduction of the medium (Lind and others 2010).

Efficient biocontrol solutions for cheesemakers could likely emerge from those recent scientific results.

Selection of nontoxigenic ripening cultures

Mold ripening cultures used to produce mold-ripened cheeses should have low toxigenic capacity (Sengun and others 2008). Variability within the *P. camemberti* species in regards to toxigenic capacity allows for the selection of weakly toxicogenic strains (Le Bars 1979). In pure cultures, all known *P. camemberti* strains are

able to produce CPA (Nout 2004); however, it is possible to select CPA-negative mutants. Consequently, selected ripening cultures were CPA-negative and only very low CPA levels were detected in Camembert-type cheeses. The same observations concerning mycotoxin production variability by *P. roqueforti* have been made (Finoli and others 2001). It was concluded that roquefortine C was the only metabolite found and that its low toxicity combined with the low levels observed make blue cheese consumption safe for consumers. Moreover, mycotoxin production can be stopped by gene inactivation of the biosynthetic pathway using mutation/selection processes or genetic engineering (Geisen and Holzapfel 1996). However, one should keep in mind that such genetically modified organism (GMO) ripening cultures are not socially accepted everywhere (Costa-Font 2011) and that special regulations could apply to the obtained products.

Common sense advice has also been expressed (Sengun and others 2008): to avoid the mycotoxin risk, cheeses with spontaneously growing molds should not be consumed.

To conclude on mycotoxin control in cheeses, we can recall that good practices in animal feeding are available to avoid mycotoxin presence in milk, and good hygiene and sanitation standards during milking, milk storage, and cheese production are required. These guidelines associated with efficient selection and preparation of ripening cultures, cold storage of cheese, as well as adapted packaging can all prevent growth of fungal contaminants.

Conclusion

Filamentous fungi encountered in cheeses may originate from raw materials such as milk or may be introduced during cheesemaking either from the environment (contamination), or deliberately inoculated using commercial ripening cultures. Contamination by filamentous fungi can be detrimental to cheese quality, causing appearance defects, off-flavors, and/or potentially toxic secondary metabolite production including mycotoxins. Mycotoxin production profiles may change under different fungal growth conditions, including substrate composition, ecophysiological factors (temperature, water activity, pH, or oxygen concentration), and biotic factors (strain-dependent mycotoxin production). To detect a wide spectrum of mycotoxins in cheese, numerous techniques are currently available. Due to the high complexity of the cheese matrix, LC/MS/MS seems to be the most sensitive technique currently available to quantify mycotoxins in cheeses.

Concerning mycotoxin toxicity, for those present in cheese, there is a lack of recent scientific data. The most common mycotoxins stable in cheese are AF, citrinin, CPA, roquefortine C, STC, and MPA. Others, including patulin, penicillic acid, and PR toxin do not persist in cheese due to the microaerophilic conditions encountered. Toxicological investigations must be made with new tools to understand the toxicity mechanisms of these mycotoxins.

Further knowledge on mycotoxin biosynthetic pathways should help to develop methodologies to elucidate the ecophysiological roles and control mycotoxin production in cheeses. The molecular basis of these pathways may also lead to complementary approaches for mycotoxin control. These guidelines, associated with efficient ripening cultures, selection of nontoxigenic strains, cold storage as well as adapted packaging conditions can all prevent the growth of fungal contaminants. This review emphasizes on future challenges that need to be addressed by the scientific community, fungal culture manufacturers, and artisanal and industrial cheese producers.

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