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Cell Cytotoxicity and Mycotoxin and Secondary Metabolite Production by Common Penicillia on Cheese Agar

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Known or potential new fungal starter culture species such as *Penicillium camemberti*, *P. roqueforti, P. nalgiovense*, *P. caseifulvum*, and *P. solitum* have been cultivated on a cheese agar medium together with the common cheese contaminants *P. commune*, *P. crustosum*, *P. discolor*, *P. atramentosum*, and *P. nordicum.* Secondary metabolites were extracted and analyzed by HPLC-DAD and tested for cytotoxicity by using the MTT-cell culture assay. Metabolites such as cyclopiazonic acid, roquefortine C, and penitrem A, previously reported from cheese, were detected together with sclerotigenin, solistatin, meleagrin, oxaline, compactins, diaportins, chaetoglobosins, rugulovasines, verrucolones, anacines, verrucines, cyclopeptines, viridicatins, and viridic acid, all metabolites not previously reported from cheese. The two *P. nalgiovense* extracts were the most toxic in the MTT-cell culture test. These extracts contained diaportines together with a number of unknown compounds. *P. roqueforti* extracts were not toxic at all. Fungal extracts from the rest of the studied penicillia were toxic at levels between these two extremes.

KEYWORDS: Associated funga of cheese; MTT-cell culture assay; mycotoxins; *Penicillium*; *P. commune*; *P. caseifulvum*; *P. camemberti*; *P. crustosum*; *P. discolor*; *P. nalgiovense*; *P. nordicum*; *P. roqueforti*; *P. solitum*; *P. verrucosum*

INTRODUCTION

Penicillium roqueforti and *P. camemberti* are widely used as starter cultures for the production of Blue and Camembert type cheeses. They are both known to produce mycotoxins, but usually only at very low concentrations or not at all at conditions for cheese production and storage (*1*, *2*). *P. camemberti* can produce cyclopiazonic acid (**Figure 1**), whereas, *P. roqueforti* has been reported to produce a number of secondary metabolites including roquefortine C, *Penicillium roqueforti* toxin (PR-toxin), mycophenolic acid, penicillic acid, penitrem A, and patulin (**Figure 1**) (*3*).

A recent study based on both chemotaxonomy and DNA fingerprinting techniques demonstrated that *P. roqueforti* should be reclassified into three species: *P. roqueforti*, *P. carneum*, and *P. paneum* (*4*). All three species produce roquefortine C, but only the two latter species are patulin producers, whereas *P. roqueforti* is the only PR-toxin producing species. These findings therefore suggest that literature reports on detection of patulin in cheese must be related to the growth of *P. carneum* or *P. paneum*. Similarly only *P. roqueforti* and *P. carneum* are mycophenolic acid producers. Cheese starter cultures usually belong to *P. roqueforti*, whereas strains of *P. carneum* and *P. paneum* are often found as contaminants of meat and bread products, respectively (*4*).

The most important mycotoxins occasionally found in milk and cheese products are aflatoxin M_1 and sterigmatocystin (**Figure 1**). Aflatoxin M_1 is a result of biotransformation of aflatoxin B_1 in cows, and sterigmatocystin is produced by *Aspergillus versicolor*, *A. nidulans*, and others (*5*). The carcinogenic mycotoxin ochratoxin A is on the other hand not considered a problem in cows milk since it is cleaved in the rumen (*6*). However, ochratoxin A and citrinin (**Figure 1**) might be produced on the surface of cheeses by penicillia during the ripening (*7*).

Recently, it was shown that ochratoxin-producing penicillia should be divided into the two species, *P. verrucosum* and *P. nordicum* (*8*). Isolates of the former species usually originate from cereals and other plant products, whereas *P. nordicum* is a frequent contaminant on protein and fat-rich products such as meat and cheese. The study showed that isolates of *P. nordicum* produce much larger amounts of OTA than isolates of *P. verrucosum* on some common synthetic media. On the other hand, only *P. verrucosum* is capable of producing citrinin (*8*).

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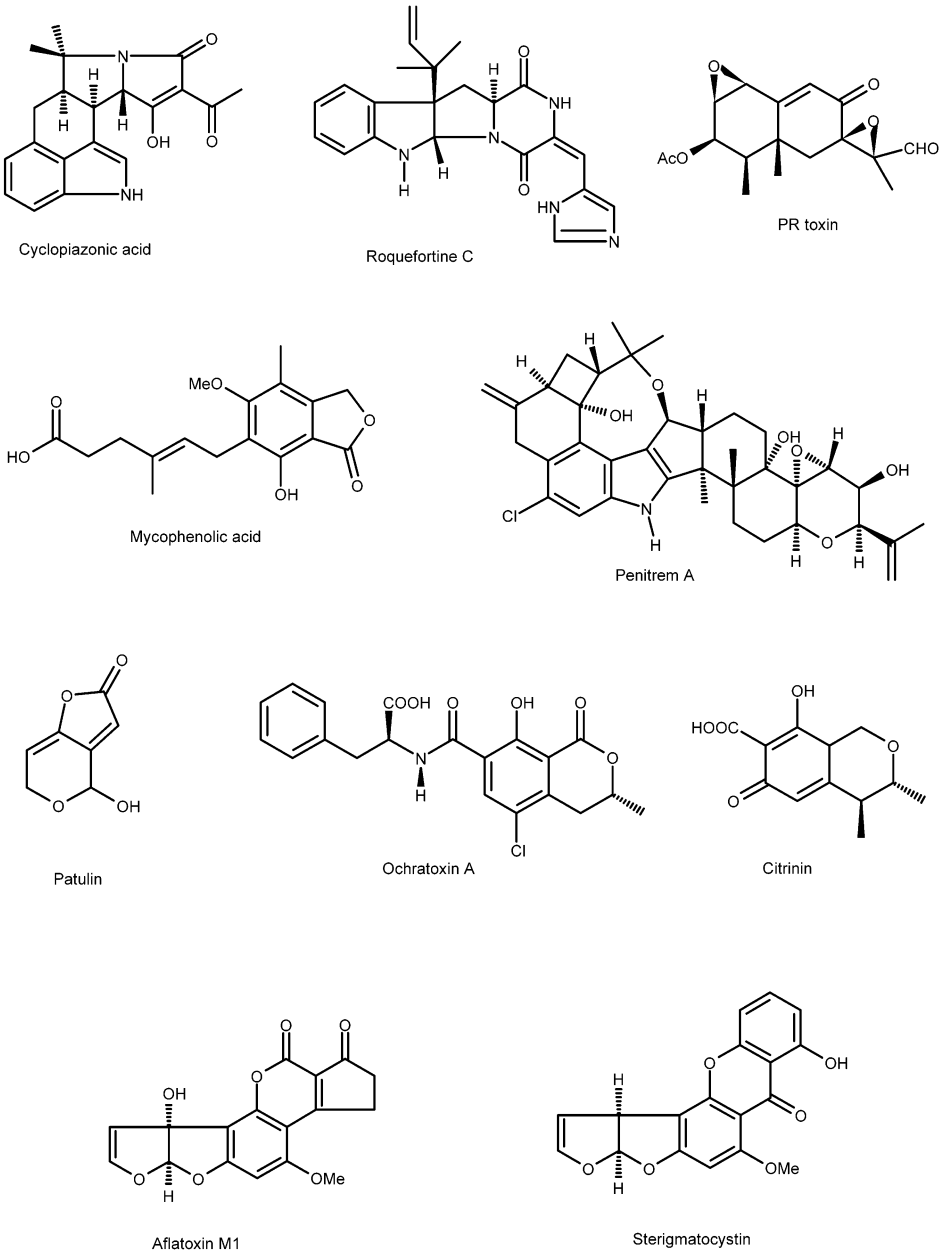

Figure 1. Important mycotoxins previously detected in cheese products.

In a recent paper describing the associated funga of soft and semisoft cheese types, *P. commune* was found to be the most important contaminant (*9*). *P. commune* is the wild type form of *P. camemberti* and is also a cyclopiazonic acid-producing species (*10*, *11*). Other known *Penicillium* species such as *P. nalgiovense*, *P. solitum*, *P. discolor*, *P. roqueforti*, *P. crustosum*, *P. atramentosum*, and *P. verrucosum* were also found frequently (*9*). The production of secondary metabolites on cheese by several of these species is, however, unknown. Lund et al. (*12*) also described a new species *P. caseifulvum* appearing as a surface contaminant of blue cheese. This species is closely related to *P. commune* and *P. camemberti*; however, it has not been shown to share their ability to produce cyclopiazonic acid.

Altogether few species represent the associated funga of cheese (*13*). Also, from several years of collaboration with different cheese-producing factories our experience is that if a factory periodically has substantial problems with fungal contamination, it is usually just one species, or even a clone (Flemming Lund, personal communication), that has been established in the particular environment.

The aim of the present study has been to qualitatively characterize both known and unknown mycotoxins and other secondary metabolites produced on cheese by some of the most important cheese-associated *Penicillium* species and to investigate their relative cytotoxicity by using a cell culture bioassay.

MATERIALS AND METHODS

Fungal Isolates. All fungal strains were obtained from the IBT Culture Collection at BioCentrum-DTU, Technical University of Denmark: *Penicillium atramentosum* (IBT: 14762, 15294), *P. camemberti* (IBT: 11567, 11569, 11755, 21603), *P. caseifulvum* (IBT: 14761, 15151, 15157, 18285, 18727, 18732), *P. commune* (IBT: 10727, 10763), *P. crustosum* (IBT: 14747, 19848), *P. discolor* (IBT: 12098, 13777, 21523), *P. nalgiovense* (IBT: 12679, 13296), *P. nordicum* (IBT: 6734, 13308), *P. roqueforti* (IBT M1, IBT M2), *P. solitum* (IBT: 13034, 14859, 15170), and *P. verrucosum* (IBT: 13077).

Medium and Culture Conditions. The cheese medium was prepared by blending blue cheese (500 g) together with 500 mL of water, containing 10 g of agar, and then melting the mixture at about 70 °C for 15 min. The cheese had ripened for 1 week at an industrial

Table 1. Fungal Metabolites Produced and Cytotoxicity Measured in Extracts of Some Important Cheese-Associated Penicillia Cultivated on a Cheese Agar Medium

species	mycotoxins and other secondary metabolites detected in fungal extracts from cheese medium	cytotoxicity of crude extracts[a]
filter control		none of 4 filters toxic
P. atramentosum	rugulovasines, meleagrin, oxaline	1, 2
P. camemberti	no known metabolites detected	2, 2, 2, 1
P. caseifulvum	rugulovasines	1, 3, 3, 2, 2, 1
P. commune	cyclopiazonic acid, rugulovasines, viridicatin	3, 3
P. crustosum	cyclopenol, roquefortine C, viridicatin, penitrem A	2, 2
P. discolor	cyclopenol, cyclopeptin, viridicatol, chaetoglobosins	2, 2, 4
P. nalgiovense	diaportins	5, 5
P. nordicum	verrucolones, anacines, sclerotigenin, viridic acid and analogues	3, 1
P. roqueforti	roquefortines A and C	both isolates not toxic at all
P. solitum	cyclopenol, cyclopeptin, dehydrocyclopeptine, viridicatins, solistatin, compactins	1, 3, 3
P. verrucosum	verrucolones, verrucines	1

[a] The isolates of the different species are mentioned in the same order as in the Material and Methods section. The cytotoxicity was measured in the MTT-cell culture bioassay and the grade of cytotoxicity of the single fungal strains is expressed with the smallest dilution step of the particular extract that showed measurable cytotoxicity. E.g. the extract from *P. atramentosum* IBT 14762 was toxic up to the first dilution step, while the *P. atramentosum* IBT 15294 extract was toxic up to the second dilution step.

factory. In this way cheese without visible growth of fungal starter culture was used. Fungal cultures were inoculated on the cheese agar plates (9 cm in diameter corresponding to a surface area of 61.25 cm^2) for 2 weeks at 15 °C on top of a thin film of cellophane that is inert to fungal digestion and allows the migration of cheese nutrients.

Extraction and Analysis of Secondary Metabolites. After 2 weeks of cultivation the cellophane films including fungal biomass were carefully transferred to glass vials and 10 mL of methanol was added to each vial. Mycotoxins and other secondary metabolites were extracted ultrasonically for 1 h before being filtered through 0.45-μm filters and taken to dryness. The extracts were redissolved in 700 μL of methanol and analyzed by HPLC-DAD according to Smedsgaard (*14*). Retention indices (RI) of fungal metabolites were calculated according to Frisvad and Thrane (*15*).

Testing of Cytotoxicity. Fungal extracts used for HPLC analysis were once again taken to dryness and sent to BAFF where they were tested for cytotoxicity by using the MTT-cell culture assay previously used to compare the relative cytotoxicity of a range of known mycotoxins (*16*, *17*). The fungal extracts (80%) were dissolved in 1 mL of cell culture medium (MEM), containing 1.7% ethanol and 0.3% DMSO giving an initial concentration of fungal extracts used in the MTT test corresponding to ca. 50 cm^2/mL. Serial log 2 dilutions of the fungal extracts (25.00, 12.50, 6.25, 3.12, 1.56, 0.78, 0.39, 0.18, 0.09 cm^2/mL) were then tested.

RESULTS

All inoculated cheese media were heavily covered with mycelium and spores after 2 weeks, and apparently the use of cellophane film had not affected the fungal growth. The HPLC analyses showed that a range of secondary metabolites could be detected in the extracts prepared from fungal mycelium (**Table 1**).

In total, the following metabolites were detected by UV and retention indices (RI) given in parentheses: anacine (790), chaetoglobosin A (1064), chaetoglobosin C (1124), compactin (1113), cyclopenol (749), cyclopeptine (867), dehydrocyclopeptine (955), cyclopiazonic acid (1109), diaportinic acid (791), diaportinol (782), dichlorodiaportine (1002), meleagrin (930), oxaline (973), penitrem A (1298), roquefortines A (829) and C (998), rugulovasines A and B (765), sclerotigenin (705), solistatin (937), verrucolone (614), verrucines A (790) and B (880), viridic acid (894) and analogues (961, 977, 1043), viridicatin (955), and viridicatol (938). All metabolites except for dehydrocyclopeptine, viridic acid (and analoges), and viridicatol were available as standards from the Mycology Group collection of more than 520 fungal metabolites.

The results of the cytotoxicity assays (**Table 1**) showed that the cellophane filters (controls) used were not toxic at all. Similarly the extracts of the two isolates of *P. roqueforti* proved to be nontoxic. Varying degrees of cytotoxicity were observed for the crude extracts of the other *Penicillium* strains tested. Among these, the most toxic extracts originated from the two *P. nalgiovense* isolates. They were both toxic in concentrations of ≥ 1.56 cm^2/mL of cell culture medium (equivalent to the fifth dilution step). One *P. discolor* extract was toxic up to the fifth dilution step. Two *P. solitum* extracts, two *P. commune* extracts, two out of six *P. caseifulvum* extracts, and one *P. nordicum* extract were toxic up to the third dilution step. The other fungal strains showed only low cytotoxic avtivities (only toxic either to the first or the second dilution step) after growth on the cheese medium (**Table 1**).

DISCUSSION

The HPLC analyses showed that all species except for *P. camemberti* produced a number of metabolites, many of which are usually produced on synthetic media or have been reported from food stuffs (**Table 1**). This was somehow unexpected since it has often been stated that even though molds find cheese a good medium for growth, this medium does not seem adequate for the production of mycotoxins (*7*). However, the growth conditions used in this study apparently favored production of mycotoxins such as penitrem A and roquefortine C from *P. crustosum*, cyclopiazonic acid from *P. commune*, and roquefortines A and C from *P. roqueforti*, all well-known to be found on cheese products.

On the other hand, mycotoxins such as ochratoxin A and citrinin that were to be expected from *P. nordicum* and *P. verrucosum* (*8*), and mycophenolic acid and PR-toxin often produced by *P. roqueforti*, were not detected in the present study. There is evidence that the latter compound is converted to PR imine in blue cheese (*18*). *P. nordicum* and *P. verrucosum* produced verrucolones (*8*), and *P. verrucosum* also produced verrucines (*19*), whereas, *P. nordicum* IBT 6734 produced anacines, sclerotigenin (*20*), and viridic acid and some analogue compounds (**Figure 2**). The latter metabolites have never been reported previously from *P. nordicum*, but are known from *P. viridicatum* (*21*).

P. discolor, *P. solitum*, and *P. crustosum* all produced cyclopeptine and analogue benzodiazepines and the two latter

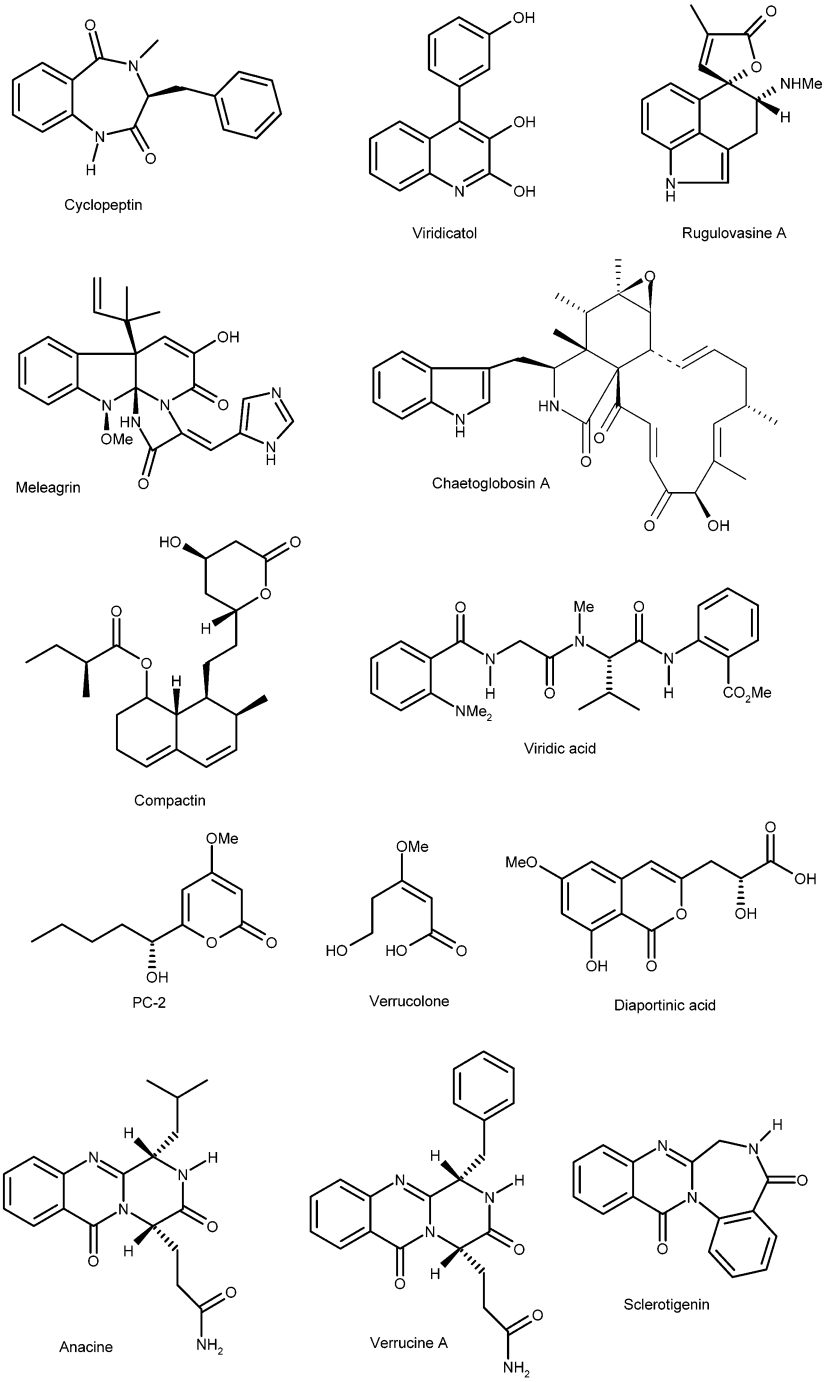

Figure 2. Fungal mycotoxins and other secondary metabolites not previously detected in cheese products.

species also shared the production of viridicatin, a metabolite also produced by *P. commune* (**Table 1**, **Figure 2**). *P. discolor* produced chaetoglobosins, and *P. solitum* produced the biologically active compactins and the related compound solistatin (*22*), all metabolites that never have been reported previously from cheese (**Figure 2**). Compactins are known for their cholesterol lowering effect (*23*).

P. atramentosum, *P. caseifulvum*, and *P. commune* produced rugulovasines A and B and also the former species produced the two related alkaloids meleagrin (**Figure 2**) and oxaline.

It is not clear which metabolites caused the two *P. nalgiovense* extracts to be the most toxic; however, the relatively large content of diaportines (*24*) (**Figure 2**) in these extracts might be the explanation. Our results are in agreement with previous studies on comparison of the cytotoxicity of *P. nalgiovense* sausage starter cultures, which also showed several *P. nalgiovense* strains to be toxic (*16*). Testing of the pure diaportine compounds should reveal whether they are the metabolites responsible for the cytoxicity observed from *P. nalgiovense* extracts.

The profiles of secondary metabolites present in the three *P. solitum* extracts were similar with respect to both the qualitative and quantitative appearance of metabolites such as cyclopenol, cyclopeptin, viridicatol, and compactins. The difference in cytotoxicity observed from these three extracts (**Table 1**) must therefore be due to cytotoxic compounds present in relatively small amounts. Similar results were observed for the two *P. nordicum* extracts and the three *P. discolor* extracts, among which one was toxic up to the fifth dilution step, whereas the two others were just toxic up to the second dilution step.

Despite containing relatively large amounts of penitrem A the two *P. crustosum* extracts were toxic only to the second

dilution step. These results are in agreement with Hanelt et al. (*17*), who concluded that the MTT assay provides only information on cytotoxic properties that are characteristic for certain, and not all compounds such as penitrem A that is a tremorgenic mycotoxin.

The extracts of the closely related species *P. camemberti* and *P. caseifulvum* gave very similar and relatively nontoxic responses in the MTT assay (**Table 1**). Also the morphology of the *P. caseifulvum* isolates resembled those of *P. camemberti* isolates, since very floccose and white mycelium could be observed from strains of both species. Since *P. caseifulvum* apparently does not produce cyclopiazonic acid (*12*), our results might indicate the potential of *P. caseifulvum* as a new starter culture for cheesemaking. These findings are in agreement with previous comparative studies (*25*), which revealed that these two species produce very similar profiles of volatile aroma compounds.

In conclusion, the present study has demonstrated that isolates of *Penicillium* species belonging to the typical associated funga of cheese are capable of producing a number of mycotoxins and other secondary metabolites not previously detected from cheese products. Also, some of the fungal extracts have been demonstrated to be relatively more cytotoxic than others. Even though the metabolites that were detected in this study were extracted from fungal mycelia, it is most likely that to some extent they also will be excreted into cheeses in in vivo situations of cheese contamination. Their possible presence in cheeses should therefore be taken into consideration especially when substantial contamination by any of the species studied here is the case. Since moldy cheeses are melted and used occasionally for production of spread cheeses, they should be examined with respect to the heat stability of these mycotoxins and secondary metabolites. With regard to food safety, the same conclusions could be drawn for fungal starter cultures that should be selected in a similiar procedure, i.e., the differentiation of secondary metabolites and in parallel the testing of cytotoxic properties.

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