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The presence of *Penicillium* and *Penicillium* mycotoxins in food wastes

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Abstract

A total of 97 samples (48 summer and 49 winter) of food waste from private households were investigated for *Penicillium* and for mycotoxins. Twenty-five *Penicillium* species were isolated and *Penicillium crustosum*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium expansum*, *Penicillium roqueforti*, *Penicillium spinulosum*, *Penicillium viridicatum*, *Penicillium commune*, *Penicillium citrinum* and *Penicillium solitum* were, in decreasing order, the most frequently identified species. Mycotoxins produced by several of these species, including mycophenolic acid, roquefortine C, penitrems A–F and thomitrems A and E, were detected. Of the 48 summer samples, 36 were severely infected and contained more than 10^5 colony forming units (CFU) *Penicillium*/g sample. The levels of mycotoxins in these samples were in the range 75–19 000 μg/kg mycophenolic acid, 40–920 μg/kg roquefortine C, 35–7500 μg/kg penitrem A, 20–2100 μg/kg thomitrem A and 20–3300 μg/kg thomitrem E. Of the 49 winter samples, only one was found to contain mycophenolic acid (4800 μg/kg) and roquefortine C (190 μg/kg), and this sample was severely infected with *P. roqueforti*. Thirty samples of food waste collected from the food manufacturing industry were also investigated. The number of *Penicillium* in these samples was between 10^5 and 10^6 colony forming units (CFU)/g sample. Seven of these samples contained mycophenolic acid ranging from 50 to 600 μg/kg and three of these samples also contained roquefortine C in the range 100–250 μg/kg.

Keywords: Food; Feed; *Penicillium*; Mycotoxins

1. Introduction

1.1. General background

In recent years, the interest in the use of biowastes such as food waste, as animal feed, especially for swine and fur farm animals, has increased. A reduction of organic waste and increased recycling of waste wherever possible have been identified as a major goal by Norwegian Government offices and plants collecting biological household waste and methods for the processing of this material to food-based liquid feed (FBL feed) have been established. Initially, food wastes from private households, commercial food outlets and the food industry were identified as possible sources of recycled feed. Food wastes are expected to be of variable hygienic quality depending on storage time and temperature as well as the type of waste and presence of bacteria and moulds. Bacteria,

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viruses, parasites and fungi are killed during the required heating process (133 °C for 30 min), but this is neither considered to be sufficient to destroy prions causing bovine spongiform encephalopathy (BSE) or scrapie nor does it degrade heat stable mycotoxins. Mycotoxins are defined as toxic secondary metabolites produced by moulds, toxic to vertebrates and other animals at low concentrations when administered by a natural route (Frisvad and Thrane, 1995). Most of the known mycotoxins are relatively stable and heat resistant and are expected to remain in the heat-treated end product. Thus, they may have an effect on animal health and possibly be of importance for consumer safety as well.

1.2. Important moulds in spoiled food

Each individual food type is normally infected by a limited number of fungi (Filtenborg et al., 1996). Food wastes, however, are very heterogeneous mixtures and reports on the combination of moulds and mycotoxins in food wastes have to our knowledge not been published. Results from previous mycological studies of food wastes in Norway have shown that *Penicillium* dominated the fungal flora (Torp and Skaar, 1998). The *Penicillium* species identified belonged to the most important mycotoxin producers known (Frisvad and Thrane, 1995) and the predominant species found was *Penicillium crustosum* (Torp and Skaar, 1998). *P. crustosum* is a very common fungus in food spoilage worldwide and several cases of animal intoxication associated with ingestion of *P. crustosum* contaminated feed have been reported (Arp and Richard, 1979; Hocking et al., 1988; Naudè et al., 2002). *P. crustosum* is a well-investigated species and its ability to form mycotoxins such as roquefortine C, penitrem A–F (De Jesus et al., 1983a,b), thomitrem A and E (Rundberget and Wilkins, 2002a) is documented.

Penicillium roqueforti and *Penicillium carneum* are two other common and well-characterised species which are known to produce the mycotoxins roquefortine C and mycophenolic acid, in addition to PR-toxin (*P. roqueforti*) and patulin (*P. carneum*) (Frisvad and Filtenborg, 1989). Other important mycotoxin-producing *Penicillium* species are: *Penicillium aurantiogriseum* (penitrem A, penicillic acid, mycophenolic acid, xanthomegnin and viomellein), *Penicillium chrysogenum* (roquefortine C and cyclopiazonic acid), *Penicillium expansum* (roquefortine C, patulin, citrinin and chaetoglobosins), *Penicillium griseofulvum* (roquefortine C, patulin and griseofulvin), *Penicillium viridicatum* (cyclopiazonic acid, penicillic acid, viomellein and xanthmegnin), *Penicillium brevicompactum* (mycophenolic acid) and *Penicillium verrucosum* (ochratoxin A and citrinin) (Frisvad and Thrane, 1995).

The purpose of this work was to examine the incidence of *Penicillium* and their mycotoxins in samples of food wastes collected from private households in order to be able to give advice regarding the use of such waste as animal feed. Additionally, we wished to study the mycotoxin concentrations in samples from food manufacturing industries, which previously had been condemned as feed because they contained higher than the recommended numbers of CFU of *Penicillium*.

2. Materials and methods

2.1. Sources of samples

Ninety-seven samples of food waste from private households were collected once in summer and once in winter from a local waste recycling plant (HRA, Hønefoss, Norway). One paper or plastic bag of waste, taken from 5 to 10 different trucks arriving at the plant, was defined as one sample. Collection of samples from different trucks gave some geographic distribution but the samples still came from a relatively limited area of the county of Buskerud. Additionally included were 30 samples collected from a plant for recycling food manufacturing waste (MEG, Tønsberg, Norway), which had been condemned for use as animal feed because they contained relatively high numbers of *Penicillium*.

2.2. Sample preparation

Each sample was transferred to a 5-l bucket and 300–500 ml of purified (grade 1) water (Purite, Oxton, England), dependent on the sample size, was added. The content of the food waste bags varied greatly, both in amount and food components. All samples were inspected and their composition recorded as they were

transferred to containers for homogenisation. Paper, bones and other components, which would interfere with homogenisation, were removed. The samples were ground and homogenised using a Wodschow and CO, H2 Handy mixer (Brøndby, Denmark).

2.3. Chemicals

Methanol, ethyl acetate, dichloromethane, acetonitrile, hexane, ammonium acetate and formic acid were of high performance liquid chromatography (HPLC) grade or p.a. and obtained from Ratburn Chemical (Walkerburn, UK). The *Penicillium* toxin standards mycophenolic acid, griseofulvin, roquefortine C, verruculogen, chaetoglobosin B, penitrem A and deuterated T-2 toxin (ISTD) were obtained from Sigma (St. Louis, USA), while penitrems B, C, D, E and F were gifts from AgResearch Ruakura (Hamilton, New Zealand) and Imperial College of Science Technology and Medicine (London, UK). Thomitrems A and E were isolated from extracts of rice inoculated with *P. crustosum*, at the National Veterinary Institute (Oslo, Norway).

2.4. Mycological investigation

Ten grams of each sample was mixed with 90 ml unbuffered peptone water (0.1% peptone and 0.85% NaCl) and blended by shaking, left for 30 min at room temperature, shaken again and dilutions from 1×10^{-1} to 1×10^{-5} prepared. From each dilution, 0.1 ml of inoculum was spread on the surfaces of duplicate plates of Malt yeast sucrose agar (MYSA), the medium routinely used at the National Veterinary Institute for counting of storage moulds (Skaar and Stenwig, 1996). The plates were incubated upside down in plastic bags in the dark for 7 days at 25 ± 1 °C before inspection and counting.

All visible colonies on duplicate plates with ≤ 150 colonies/plate were counted, also all *Penicillium* colonies and the numbers of each different *Penicillium* type. Results are calculated as weighted mean number and given as the number of colony forming units per gram of sample (CFU/g).

Each type of *Penicillium* colony was subcultured onto Malt extract agar (MEA), Czapek yeast extract agar (CYA) (Samson and van Reenen-Hoekstra, 1988), Yeast extract sucrose agar (YES) (Samson et al., 1996), Creatine sucrose agar (CREA) (Samson and van Reenen-Hoekstra, 1988) and Nitrite sucrose agar (NSA) (Frisvad, 1981). Plates were three point inoculated and MEA, CYA and YES incubated for 7 days at 25 °C, CREA and NSA for 7 days at 20 °C.

The isolates were identified according to Pitt (1979), Frisvad and Filtenborg (1990), Pitt and Cruickshank (1990) and Samson et al. (1996). For identification of *Penicillium commune* and *Penicillium palitans*, filter paper method and thin layer chromatography were used according to Lund (1995a,b).

2.5. Chemical analysis

For extraction, clean up and chemical analysis, a modified method described by Rundberget and Wilkins (2002b) was used. One part of the sample was freeze-dried and 25 g of this was extracted with 100 ml acetonitrile–water (9 + 1, v/v) for 5 min on a T25 Ultrathurax (Janke and Kunkel, Staufen, Germany). After the addition of 50 ml of hexane, the sample was shaken for 30 min. The hexane layer was discarded and the extract filtered (Schleicher and Schuell 520 B 1/2 folded filters). The extract (5 ml) was evaporated under a gentle stream of nitrogen and the residue dissolved in 700 μl of methanol. After dilution with 300 μl of water, the sample was defatted once more with 1 ml of hexane before the methanol–water fraction was filtered using a Spin-X centrifuge 0.2 μm nylon filter (Costar, Corning, USA). Chromatography was performed on a Symmetry C-18 column (5 μm, 4.6 × 150 mm) (Waters, Milford, USA), using a model P4000 pump and a model AS3000 auto sampler (TSP, San Jose, USA). To analyse mycophenolic acid, roquefortine C, griseofulvin, chaetoglobosin B, verruculogen and penitrem A, a gradient system of methanol–water containing 0.005 M ammonium acetate (~ pH 6) was used as mobile phase. The program started at 40% methanol, then the percentage was raised to 90% in 20 min. To analyse the different penitrems (A–F) and thomitrems (A and E), a gradient system of acetonitrile–water containing 0.005 M ammonium acetate was used as mobile phase. The program started at 40% acetonitrile, then the percentage was raised to 90% in 15 min. In both methods, the flow rate was 0.7 ml/min and 20 μl of sample was injected. The high performance liquid chromatography (HPLC) system was coupled to mass spectrometer (MS), an ion

trap analyser, with an atmospheric chemical ionisation (APCI) interface (LCQ, Finnegan MAT, San Jose, USA). The instrument was operated in MSMS single reacting monitoring (SRM) for the detection of mycophenolic acid, roquefortine C, griseofulvin, chaetoglobosin B, verruculogen and penitrem A and MS full scan positive ion mode for detection of the penitrems and thomitrems. The ion injection time was set to 300 ms with a total of 3 microscans/s. For APCI, a vaporisation temperature at 350 °C, a sheath gas rate at 25 units nitrogen (1 unit equals approximately 10 ml/min), an auxiliary gas rate at 5 units nitrogen, a corona discharge voltage of 4.5 V and a capillary temperature of 200 °C were used. Limit of detection was 20–75 μg/kg depending on the toxin.

3. Results

3.1. Household samples

The mean *Penicillium* count was 6.5×10^6 CFU/g for summer samples and 1.6×10^4 CFU/g for winter samples. Twenty-five *Penicillium* species were isolated, the most frequently identified in decreasing order of frequency being *P. crustosum*, *P. brevicompactum*, *Penicillium spinulosum*, *P. chrysogenum*, *P. expansum*, *P. roqueforti*, *Penicillium olsonii*, *P. viridicatum*, *P. commune*, *Penicillium citrinum* and *Penicillium solitum*. Mean counts of all species recovered are given in Table 1. The same *Penicillium* species were dominant in both summer and winter samples but the prevalence

Table 1
Penicillium species demonstrated in samples of food waste from private households

Species	Summer (%)	Mean (CFU/g)	Winter (%)	Mean (CFU/g)	Mycotoxins reported[a]	Food source reported[a]
P. crustosum	52	6.3×10^6	12	2×10^3	Roquefortine, penitrems	Cereals, nuts, fruits, meat, egg
P. brevicompactum	40	1.8×10^6	34	3×10^3	Mycophenolic acid	Cereals, meat, fruits, vegetables
P. spinulosum	29	2.6×10^6	36	2×10^4		Fats, oils
P. chrysogenum	21	1.8×10^6	8	2×10^4	Roquefortine	Meat, cereals, egg, fruits, cheese
P. expansum	17	5.0×10^5	12	2×10^3	Roquefortine, patulin, chaetoglobosin	Fruits, vegetables, meat
P. roqueforti	15	3.1×10^6	10	8×10^{3b}	Mycophenolic acid, roquefortine, PR-tox	Cheese, fruit, bread
P. olsonii	15	1.3×10^6	8	2×10^3	Verruculone	Tomatoes, meat, beans
P. viridicatum	14	2.6×10^6	10	2×10^3	Xanthomegnin, penicillic acid	Cereals
P. commune	13	1.6×10^6	4	7×10^2	Cyclopiazonic acid	Cheese, nuts, meat, fats
P. aurantiogriseum	8	3.6×10^5	2	1.3×10^5	Verrucosidin, penicillic acid	Cereals, meat, fish
P. solitum	4	2.1×10^5	8	2×10^3	Viridicatins	Fruits, nuts, fish, cheese, meat
P. verrucosum	4	3×10^4	2	2×10^3	Ochratoxin, citrinin	Cereals, meat, fish
P. citrinum	2	1.8×10^5	12	5×10^3	Citrinin	Nuts, cereals, fruits
P. janthinellum	2	1.8×10^6	0	0	Janthitrems	
P. echinulatum	2	4.5×10^5	2	3×10^3	Territrems, viridicatins	Nuts, fats,
P. discolor	2	9×10^4	0	0	Viridicatins	Nuts, cheese
P. carneum	2	1×10^4	2	3×10^4	Mycophenolic acid, roquefortine, patulin	Cereals, bread, meat
P. purpurescens	2	9×10^3	0	0		Cereals
P. italicum	2	2×10^3	6	9×10^3	Deoxybrevinamide E	Fruits, vegetables
P. digitatum	0	0	2	1.2×10^6	Tryptoquivalins	Citrus fruits
P. hirsutum	0	0	2	5×10^4	Roquefortine	Cereals, fruits
P. oxalicum	0	0	2	1×10^4	Roquefortine	Nuts
P. corylophilum	0	0	2	2×10^3		Meat, egg, cereals, bread
P. citreonigrum	0	0	2	5×10^2	Citreoviridin	Rice
P. resticulosum	0	0	2	5×10^2		

Occurrence and mean mould counts (CFU/g) of 48 summer and 49 winter samples. Common food sources and mycotoxins produced are given for the species found.

[a] After Frisvad and Filtenborg, 1989; Frisvad and Samson, 1991; Filtenborg et al., 2000; Pitt, 1979.

[b] One sample with 2.9×10^8 CFU/g is excluded and not represented in the given mean value.

of *P. crustosum* was significantly higher in the summer than the winter samples (52% compared to 12%). *P. crustosum* was found in 25 of the 48 summer samples and in 12 was also quantitatively dominant.

Only 1 of the 49 samples collected in winter (February) contained mycotoxins, whereas 36 of the 48 collected in summer (August) contained at least 1 of the 13 mycotoxins analysed for (Table 2). Levels of mycotoxins in these samples were in the range 75–19 000 μg/kg mycophenolic acid, 70–920 μg/kg roquefortine C, 35–7500 μg/kg penitrem A, 20–2100 μg/kg thomitrem A and 20–3300 μg/kg thomitrem E. Two samples contained 60 and 250 μg/kg chaetoglobosin B and two 70 and 390 μg/kg griseofulvin.

Thirty-two (70%) of the samples collected in summer had high mould counts ($>1 \times 10^5$ CFU/g) and detectable amounts of mycotoxins. In 22 (46%) of the summer samples containing mycotoxins, the mould counts were higher than 1×10^6 with only four samples with counts lower than 1×10^5 CFU/g found to contain mycotoxins. *P. crustosum*, *P. brevicompactum* and *P. spinulosum* were the dominant species. Except for three samples, one with high numbers of *P. roqueforti*, one with high numbers of *P. digitatum* and one with high numbers of *P. expansum*, the winter samples had 100–1000 times lower mould counts than the summer ones. The one sample with 2.9×10^8 CFU/g *P. roqueforti* was the only winter sample that contained detectable mycotoxins, 4800 μg/kg mycophenolic acid and 190 μg/kg roquefortine C. More than one *Penicillium* species was demonstrable in all samples, both summer and winter. Fourteen (30%) of the summer samples were severely infected, $>1 \times 10^5$ CFU/g by three or four different species but in most of these one of the species was dominant and typically had a tenfold higher count than the other species.

Table 2
Mycotoxins (μg/kg) found in samples of food waste from private households

Mycotoxin	Summer		Winter	
	$n=48$	μg/kg	$n=49$	μg/kg
Mycophenolic acid	36	75–19 000	1	4800
Griseofulvin	2	70–390		
Roquefortine C	13	70–920	1	190
Chaetoglobosin B	2	65–250		
Verruculogen		–		
Penitrem A	11	35–7500		
Penitrem B	5	20–450		
Penitrem C	2	35–60		
Penitrem D	5	20–620		
Penitrem E	1	70		
Penitrem F	2	30–60		
Thomitrem A	11	20–2100		
Thomitrem E	11	20–3300		

3.2. Food manufacturing industry samples

Seven of the 30 samples from the food manufacturing industry contained mycotoxins. All contained mycophenolic acid in the range 50–600 μg/kg and three of these additionally contained roquefortine C in the range 100–250 μg/kg. None of the other toxins analysed for were detectable.

4. Discussion

High levels of *Penicillium* and detectable amounts of mycotoxins were demonstrated in many samples and the results of this study raise matters of serious concern regarding the use of food wastes for animal feed. Several authors have suggested that feed is considered to be of good hygienic quality if the mould counts are less than 3×10^4 CFU/g, of acceptable quality if mould counts are less than 1×10^5 CFU/g and have unacceptable quality if mould counts are higher than 1×10^5 CFU/g (von Flatscher and Willinger, 1981; Stenwig and Liven, 1988). According to these guidelines, on *Penicillium* counts only, 70% of the summer samples and 10% of the winter samples investigated were of unacceptable hygienic quality. The real mould counts were however probably even higher since, in the present study, other fungal genera also growing on the material were not taken into consideration. In addition to the high mould counts of *Penicillium*, several *Penicillium* species, which are regarded as toxigenic, were demonstrated in the samples.

P. crustosum, the species demonstrated most frequently and also in high counts, has the advantage of being a good spore producer and under favorable growing conditions a vast number of spores may be produced. *P. spinulosum* was also frequently found both in summer and winter. Other important species

occurring in the samples were *P. expansum*, which is usually found on vegetables and fruits, *P. brevicompactum* and *P. chrysogenum*, which are very common in indoor environments because of their ability to grow at low water activities, and *P. roqueforti*, which often occurs on cheese products and contaminates other refrigerated products (Filtenborg et al., 1996). *P. citrinum* is considered to be one of the most common fungi worldwide, occurring in all kinds of foods and feeds and in almost all climates (Pitt, 2002). *P. citrinum* was demonstrated in 2% of the summer samples and in 14% of the winter samples. The mould counts of *P. citrinum* were very low (mean = 5000 CFU/g) and it is most likely that no mycotoxins were produced by this species or that they were produced at levels lower than the limit of detection (20 μg/kg).

The samples were subjected to a brief visual inspection prior to examination and some connections between food type in the waste and *Penicillium* species found could be made. Samples that contained much bread were usually contaminated by *P. brevicompactum* and *P. crustosum*, while samples that contained much vegetable material were in many cases contaminated by *P. expansum*. *P. expansum* is a well-known producer of the mycotoxin patulin, however, no method for analysing this toxin in these types of samples was available.

Eleven of the summer samples contained penitrems and thomitrems. All these samples were heavily infected by *P. crustosum* and we suggest that the penitrems and the thomitrems were produced by this species. Of the 13 summer samples that contained roquefortine C, most samples were infected not only by *P. crustosum* but also by *P. expansum*, *P. chrysogenum* or *P. roqueforti*, which all are known roquefortine C producers. Thus, we cannot determine which species produced the roquefortine C. Mycophenolic acid was found in all the 36 toxins containing summer samples. *P. brevicompactum* and *P. roqueforti* are known producers of mycophenolic acid, but because of the large number of samples that contained the toxin, several which were not infected with either of these two *Penicillium* species, it is likely that other *Penicillium* species can produce mycophenolic acid. Only a few samples were infected with low levels of *P. verrucosum*; hence, no effort was made to analyse these samples for ochratoxin A.

In the 30 samples originating from the food manufacturing industry, seven were found to contain mycotoxins. These samples were included in our study because they had previously been found to be heavily contaminated by fungi with fungal counts between 10^5 and 10^6 CFU *Penicillium*/g. However, in these samples, the fungi were not identified to species, but since only mycophenolic acid and roquefortine C were found in these samples, it is likely that they were infected with *P. brevicompactum*, *P. expansum*, *P. chrysogenum* or *P. roqueforti* rather than with *P. crustosum*.

Penicillium species have been reported to produce several hundred different toxic components. Only 13 different mycotoxins were searched for in this investigation. A broader search for mycotoxins might have detected more than these reported.

Feeding mycotoxin-contaminated food to pigs may cause accumulation of mycotoxins in the tissues. There are several surveys that have shown the presence of another *Penicillium* mycotoxin, ochratoxin A, in pig blood. Ominski et al. (1996) found that 36% of the serum samples taken from 1600 pigs in Canada contained detectable amounts of ochratoxin A, while Langseth et al. (1993) investigated serum samples from 216 Norwegian pigs and found that 5% of the samples contained ochratoxin A. It has been shown that ochratoxin A takes as long as 14 days to be completely excreted from muscle tissue of pigs (Humphreys, 1988). Other *Penicillium* mycotoxins such as cyclopiazonic acid need 3–4 days to be excreted completely from muscle tissue of rats (Norred, 1990), while citrinin and penitrem A are excreted from plasma and muscle tissue of pigs and sheep after only a few hours (Sandor et al., 1991; Laws et al., 1987). It cannot be excluded that some of the mycotoxins found in this study could have the ability to accumulate in pig tissues and be a possible health risk to man.

No instructions are given for storage time and hygienic quality of organic food items being disposed of by private households and these could have varied greatly. During summer (August), the outdoor temperatures are 15–20 °C, whereas in winter (February), they are around or below 0 °C. The food wastes were probably stored indoors at 20–25 °C for some time before being put in garbage cans. Storage in garbage containers outside in wintertime would reduce growth

significantly, explaining the differences between summer and winter mould counts and mycotoxin levels. The containers were collected only once a week, hence increasing the risk of fungal growth especially in the summer.

Demands from the authorities for a hygienically safe raw material for feed are hardly fulfilled by the existing system of storage and collection. The Norwegian authorities state that raw materials for feed shall be clean, healthy and of hygienic quality, and not entail risk of damaging animals, humans or the environment (Ministry of Agriculture, 2002). On our findings, it is not recommended, with the current procedures of sorting and collection, that food wastes from private households should be used as animal feed. At a minimum, to improve the hygienic quality of wastes from private households, there must be stricter instructions regarding the quality and the type of food items being disposed of, together with more frequent collection than is currently used. As shown in this study, there is not always a good correlation between fungal counts and the concentration of detected mycotoxins in a sample. Most of the samples that contained mycotoxins had a mould count above 1×10^6 CFU/g but there were a few samples with relatively low mould count, below 1×10^5 CFU/g, that contained significant concentrations of mycotoxins. It is difficult to give recommendations for what is acceptable hygienic quality of food waste. Nevertheless, recommendations should be based on the type of moulds present and fungal counts as mentioned above. Food waste samples originating from the food industry are subject to strict regulations regarding storage temperature, which must be lower than 5 °C. The food wastes from the food manufacturing industry were found to be of a much better hygienic quality than those from households but should still be monitored regularly both for the presence of fungi and mycotoxin.

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