



Distribution of mycotoxins produced by *Penicillium* spp. inoculated in apple jam and crème fraiche during chilled storage

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ARTICLE INFO

Keywords:

Patulin
Ochratoxin A
Citrinin
Roquefortine C
Exposure

ABSTRACT

Estimations of consumer exposure to mycotoxins through surveillance of mycotoxins in the food trade are well described, but the exposure due to mouldy food in private homes is not known, and may result from removing visible mould on food and eating the rest. In this study, we followed the growth of *Penicillium expansum* on the surface of apple jam and *Penicillium verrucosum* on crème fraiche, as well as production and distribution of fungal metabolites throughout the sample (approx. 6 cm high divided into three equal layers), using a multianalyte method, over time (up to 28 days) and at 4, 8 and 15 °C.

Growth rates and apparent lag times for *P. expansum* in apple jam at different temperatures were estimated by fitting to the Baranyi model. The growth rates were 1.7, 2.7 and 4.3 mm day⁻¹ for storage at 4, 8 and 15 °C, respectively; apparent lag times decreased with increasing storage temperature and were 10.6, 7.9 and 2.6 days at corresponding temperatures. Patulin and roquefortine C were identified and quantified, among other fungal metabolites. Patulin was detected in all 2-cm layers of the apple jam at 15 °C. Concentrations in the upper two layers of the jar corresponded to exposures exceeding the health based guidance value (HBGV) for a normal serving size. Consequently, removal of the mouldy part is insufficient to avoid unhealthy exposure. In contrast to patulin, roquefortine C was also produced at 4 °C.

The growth of *P. verrucosum* on crème fraiche was very restricted and could not be modelled. Despite the small colony (8 ± 0.5 mm in diameter), ochratoxin A and citrinin were detected after 21 days at 15 °C in the top 2 cm layer (including the fungal colony), and at concentrations in a normal serving corresponding to an exposure above the HBGV established by EFSA for both mycotoxins. Questiomycin A, an antibiotic, was also produced in crème fraiche but in contrast to the two mycotoxins, was detected throughout all layers of the crème fraiche and was produced also at 4 and 8 °C.

As a complement to a previous study, we also present production and the distribution of major fungal metabolites in apple jam and crème fraiche for some additional fungal strains (*P. crustosum*, *P. roqueforti* and *P. verrucosum* on apple jam and *P. expansum* on crème fraiche). A pilot study investigating the effect of inoculation size on toxin production may have implications for the best inoculum to use in experimental studies.

1. Introduction

Consumers are exposed to various levels of mycotoxins in their diets, and the exposure from food moulded while stored at home is unknown. A common question from consumers to the National Food Agency is if food that has become mouldy after purchase can be salvaged by removing the mouldy part, or if they should discard it entirely. A limited study (n = 33) by [Terpstra et al. \(2005\)](#), which cannot be extrapolated to the whole Dutch population, examined the consumer

behaviour and knowledge concerning food storage and disposal. Mould on cheese was most frequently mentioned (15 out of 28) as the reason to get rid of the product. The consumers' knowledge of food storage did not correspond with their actual behaviour, i.e. stored longer than the recommended guidelines for leftovers, fruit juice and cheese. More recently, [Sanders et al. \(2015\)](#) performed a quantitative survey (n = 520) in the Flemish population concerning the awareness about contamination of food and feed with mycotoxins. Most people were aware of the negative effect of moulds in food and feed and around 60%

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<https://doi.org/10.1016/j.ijfoodmicro.2018.12.003>

Received 20 September 2018; Received in revised form 3 December 2018; Accepted 6 December 2018

Available online 07 December 2018

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picked out moulds as the producers of mycotoxins, but the majority did not have an idea about the toxicity of these toxins and the potential effects are better known for bacteria than for moulds. The authors concluded that more awareness concerning mould growth, production of mycotoxins and their toxic effects on organisms, exposure pathways and how to deal with mouldy food is of high value and importance for the population, and that more information is needed to increase awareness. Today, many consumers are aware of the need to minimise food waste, for sustainability and economic reasons, and may remove the visibly mouldy portion and eat the rest. Reviewing the literature showed that there are a limited number of investigations reporting on the distribution of mycotoxins in mouldy food; most of the studies are old and did not cover the range or concentrations of mycotoxins that we know today (Olsen and Svanström, 2017). Akerstrand and Andersson (1979) studied mould growth and formation of patulin in apple jams with different concentrations of added sugar (low and high, corresponding to 25 or 33 or 100 g sugar per 100 g jam) and with or without added preservative (sodium benzoate). The jams were kept at cool temperature (15 °C) and inoculated with *P. expansum*. After 15 days patulin had reached the bottom of the jar and but no correlation was observed between colony growth and patulin production. Mould growth was observed in 1 out of 4 jams with high sugar content but no patulin was detected. Sodium benzoate reduced mould growth but had a stimulating effect on patulin production at low sugar content. At that time, no other fungal metabolites were identified. Patulin diffusion has also been studied in blackberry, blueberry and strawberry jams by Lindroth et al. (1978) who also found a strong but not complete inhibition of patulin formation when adding 44 g sugar per 100 g jam. Highest levels of patulin were found in jams without added sugar. The same research group also studied aflatoxin production in jams with or without added sugar. Aflatoxin was only found in jams without added sugar and which were stored at room temperature (Pensala et al., 1978). Similar results were also obtained by Ostry et al. (2004) in apricot jam. The highest levels of aflatoxins were formed at room temperature but concentrations up to 25 µg/kg of aflatoxins could be found on the surface of the jam stored at 6 °C.

Fresh milk seldom has spoilage with moulds, but milk products such as yoghurt and crème fraîche can be contaminated with *Penicillium* species while stored refrigerated (Pitt and Hocking, 2009). Mould growth and toxin production in these products are very few, but Sulyok et al. (2010) found very high levels, 300–16,000 µg/kg, of ochratoxin A in spontaneously mouldy crème fraîche. They also reported 49 different fungal metabolites in 87 mouldy food samples collected from private homes, being the first report of the natural occurrence of many of these metabolites in foods. To be able to give sound advice to consumers, we initiated a study (Olsen et al., 2017) where we inoculated different fungal strains on food items (hard cheese, crème fraîche, tomato purée, apple and blueberry jam). The outcome showed that mouldy food can contain a cocktail of bioactive secondary metabolites including mycotoxins and sometimes at concentrations which could imply a health risk. Measurements of the distribution of fungal metabolites at different distances from the colony on the food surface showed that the fungal metabolites were not detected > 2 cm into the inner core of the hard cheese (Gouda). On the other hand, in more liquid foods, such as crème fraîche, fruit jams and tomato purée, toxins were distributed throughout the entire food sample. In this study, we continue to study the distribution of fungal metabolites from additional psychrotrophic *Penicillium* species and follow growth and mycotoxin production over time in apple jam and crème fraîche stored at cool or refrigerated temperatures (4 to 15 °C).

2. Materials and methods

2.1. Fungal strains and spore suspensions used for inoculum

The following fungal species were used as inoculum on the different food commodities: *Penicillium expansum* (M17, gruel powder); *P.*

roqueforti (M19, gruel powder); *P. crustosum* (M55, sunflower seeds); *P. nordicum* (M40, cheese); *P. verrucosum* (OTA16 and OTA21, cereal grain). Identification codes for the internal fungal collection at the National Food Agency are shown within brackets together with original isolation source.

The toxin production of each species additional to those of the previous study (Olsen et al., 2017) was examined by growth on Yeast Extract Sucrose agar (YES) plates at 25 °C for 7 days. Agar plugs (n = 5) were taken from each colony and sent to the laboratory at IFA-Tulln in Austria (Section 2.4).

Spore suspensions of each species for inoculation of food items were prepared as follows: a pure culture of the strain was streaked onto malt extract agar slants (200 ml in 500 ml flasks). Following incubation at 25 °C for 7 days, the spores were harvested in 0.1% peptone solution containing 0.015% Tween 80 (822187, Merck, Darmstadt, Germany) using a sterile plastic loop. The crude suspension was filtered through sterile gauze fabric into sterile glass bottles after which the spore concentration was determined microscopically using a Burkler chamber; the absence of mycelial fragments in the suspension was also confirmed microscopically.

From the original suspension, a suspension with a spore concentration of 2500 spores in 5 µl was prepared as inoculum for immediate use in the food inoculations studies. Another spore suspension of 5000 spores per 5 µl was prepared for the study of the effect of inoculation size. This suspension was diluted 1:10, 1:100, and 1:1000 in peptone solution.

2.2. Food items

The following food items were obtained from a local shop: 250-ml light crème fraîche (15% fat, modified maize starch, pectin and locust bean gum as thickening agent, n = 44); and 375-gram organic apple jam (26% added sugar, pectin, citric acid, ascorbic acid, potassium citrate, n = 23) of the same batch. The light crème fraîche and apple jam were of the same brands as used in the previous study (Olsen et al., 2017) and the a_w of these products was 0.98 and 0.96, respectively.

The apple jam, 172 ± 5 g, was transferred to sterile plastic beakers and covered with sterile foil. The crème fraîche samples were covered with the original plastic lids which were perforated three times with a sterile needle to allow air into the jar.

Uninoculated samples of each food item served as controls and were kept frozen (−18 °C) for chemical analyses.

2.3. Experimental design

2.3.1. Distribution of mycotoxins and other major secondary metabolites in apple jam or crème fraîche

The fungal spore suspensions were inoculated on the top centre part of each food item by placing 5 µl (2500 spores) with a micropipette on the surface. All inoculations were carried out in duplicate. The following fungal species were inoculated on the following food items: *P. expansum* (M17), *P. nordicum* (M40), and *P. verrucosum* (OTA21) on crème fraîche; *P. roqueforti* (M19), *P. crustosum* (M55), and *P. verrucosum* (OTA16) on apple jam.

All samples were incubated at 15 °C for 14 days. The colony diameter was measured and all samples were placed in a freezer (−18 °C) before further processing. The frozen samples were divided into subsamples by cutting parallel with the top surface at 2 cm and 4 cm from the top, yielding three subsamples called layers A, B, and C. The uppermost subsample A included the surface part of the fungal colony. Each individual subsample was weighed. Finally, the subsamples were thawed approx. 30 min before homogenization with a stick blender for 1–2 min. Approximately 5 g of each homogenized subsamples was weighed in a 50 ml-Falcon tube and stored at −18 °C until the chemical analyses (see Section 2.4 Analyses of mycotoxins and other fungal metabolites).

The production of secondary metabolites was expressed as in the previous study (Olsen et al., 2017) as concentration in layer A, B and C (μg metabolite/kg).

2.3.2. Mould growth, production and distribution of mycotoxins in apple jam and crème fraiche depending on temperature and time

Apple jam: *P. expansum* (M17) was inoculated on the top centre part of 36 samples by placing 5 μl (2500 spores) with a micropipette on the surface. Twelve samples were incubated at each temperature, 4, 8 and 15 °C. Triplicates of samples were taken out at four different storage times: at 15 °C, samples were taken at 3, 7, 10 and 14 days after inoculation; at 8 and 4 °C, samples were taken at 3, 7, 14 and 21 days. The colony diameter was measured and samples were placed in a freezer (−18 °C) before division, homogenization, and analyses as described above (Section 2.3.1).

Crème fraiche: *P. verrucosum* (OTA16) was inoculated on the top centre part of 36 samples by placing 5 μl (2500 spores) with a micropipette on the surface. Twelve samples were incubated at each temperature, 4, 8 and 15 °C. Triplicate samples were taken at four different times: at 3, 14, 21 and 28 days after inoculation. The colony diameter was measured and samples were placed in a freezer (−18 °C) before division, homogenization, and analyses as above (Section 2.3.1).

2.3.3. Effect of the inoculation size on growth and production of fungal metabolites

Four apple jam samples were inoculated with 5, 50, 500, and 5000 spores of *P. expansum* (M17). The samples were incubated at 15 °C for 14 days. The colony diameter was measured after 3, 5, 7, 10, 12 and 14 days. After 14 days, the whole sample (170 \pm 10 g) was homogenized as above and a subsample (approx. 5 g) was weighed into a 50 ml-Falcon tube and stored at −18 °C until the chemical analyses.

2.4. Analyses of mycotoxins and other fungal metabolites

All samples were extracted using 4 ml of extraction solvent per gram sample. For agar plugs the extraction solvent that was found to be the best compromise (acetonitrile/water/acetic acid 79 + 20 + 1, v + v + v) by Sulyok et al. (2006) was used; however, the acetonitrile fraction was increased to 84% (v/v) for the food samples to take into account their water content, as the extraction solvent has been optimized for dried grains [water content < 14%, which is significantly lower than the water content in YES-agar plugs (82% water)]. The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). The extracts were transferred into glass vials using Pasteur pipettes and aliquots of 500 μl were diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20 + 79 + 1, v + v + v). After appropriate mixing, 5 μl of the diluted extract were injected into the LC-MS/MS system without further pretreatment. For each sample type, three non-inoculated replicates were spiked and subsequently analysed to determine the apparent recoveries.

Detection and quantification were performed as described by Malachova et al. (2014). Briefly, a QTrap5500 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and 1290 Series UHPLC System (Agilent Technologies, Waldbronn, Germany) was used. Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150 \times 4.6 mm i.d., 5 μm particle size, equipped with a C18 security guard cartridge, 4 \times 3 mm i.d. (all from Phenomenex, Torrance, CA, US). ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs. The sMRM detection window of each analyte was set to the respective retention time \pm 27 s and \pm 42 s in positive and in negative mode, respectively. The target scan time was set to 1 s. Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid, that each exhibit

only one fragment ion), which yields 4.0 identification points according to Commission decision 2002/657/EC. Quantification was based on linear, 1/x weighed calibration using serial dilutions of an external multicomponent stock solution. Concentrations were corrected for apparent recoveries. The accuracy of the method was previously verified by participation in inter-laboratory comparison studies (De Girolamo et al., 2017; Malachova et al., 2014) including a regular scheme organized by BIPEA (Gennevilliers, France). Limits of detection and limits of quantification were determined following the EURACHEM guide (Magnusson and Örnemark, 2014).

2.5. Modelling mould growth

The observed growth of mould colonies on the upper food surface was expressed as average colony diameter along two perpendicular axes. The mean colony diameters over time, t (days), were fitted to a biphasic Baranyi model (Eq. (1)), described in Marín et al. (2008, IJFM). The growth rate (μ) and the apparent lag time (λ , approximating time to visible growth) were estimated using the nls function in the statistical and programming software R (R Core Team, 2016).

Colony diameter (mm)

$$= \mu \times \left(t + \frac{1}{\mu} \times \log \left[e^{(-\mu \times t)} + e^{(-\mu \times \lambda)} - e^{(-\mu \times t - \mu \times \lambda)} \right] \right) \quad (1)$$

2.6. Exposure assessment

To assess the risk of different concentrations of mycotoxins in apple jam and crème fraiche the following consumption data was used.

Data on portion sizes of apple jam was collected from the Swedish national survey Riksmaten adults (NFA, 2012). The average portion size was 39 g, the median 51 g, the 50th percentile 34 g and the 95th percentile 85 g ($n = 240$). There were no portion sizes for crème fraiche available in the national survey, instead the mean of 10 randomly picked recipes including crème fraiche was used and the median portion size of crème fraiche was 50 \pm 12 g.

The body weight (b.w.) for an adult person was set to 70 kg.

3. Results and discussion

The capacity of the fungal isolates to produce secondary metabolites, including mycotoxins, was confirmed by first growing the isolates on YES-agar plates, a methodology commonly used for checking mycotoxin production of fungal strains. The results are included in the previous study (Olsen et al., 2017). In this study, we added a few other strains with ochratoxin A producing capacity, one *P. nordicum* (M40) and two *P. verrucosum* (OTA16 and OTA21), which were checked using the same methods as in the previous study. The secondary metabolite patterns of those additional fungal strains are presented in Table 1.

3.1. Distribution of mycotoxins and other major secondary metabolites in apple jam or crème fraiche

The first trial is a continuation of the investigation (Olsen et al., 2017) to study fungal growth and distribution of fungal metabolites in apple jam and crème fraiche. In total, 28 different metabolites were detected in the two foods. Not all these fungal metabolites are shown in Table 2, only those which were identified as metabolites originating from the fungal strains used in this study or by Olsen et al. (2017) and that are regarded as mycotoxins or biologically active compounds used in the development of drugs.

The isolates of *P. nordicum* (M40) and *P. verrucosum* (OTA21) produced very few metabolites and no mycotoxins in crème fraiche during the 14 days incubation at 15 °C and consequently these results are excluded from Table 2. The *P. verrucosum* strain (OTA16) did not produce

Table 1

Secondary metabolite pattern of fungal isolates. Metabolites regarded as mycotoxins according to Bråse et al. (2009) are in bold letters.

Fungal metabolite ^a	<i>P. nordicum</i> (M40) ^b	<i>P. verrucosum</i> (OTA16)	<i>P. verrucosum</i> (OTA21)
Anacin	1506	< LOQ ^c	< LOQ
Citrinin	< LOQ	49,597	< LOQ
Ochratoxin A	224	1085	108
Ochratoxin B	132	395	< LOQ
Pestalotin	98,520	24,488	82,593
7-Hydroxypestalotin	48,855	25,330	95,744
Questioniomycin A	13,347	7169	11,643

^a Expressed as total ng in YES-agar plugs.

^b Internal culture collection identification code.

^c Limit of quantification.

ochratoxin A or citrinin in apple jam but did produce the fungal metabolite questioniomycin A, which was even detected in the bottom layer C. Questioniomycin A is an antibiotic (phenoxazine) produced by several *Streptomyces* species and some fungi and bacteria. Questioniomycin A is weakly active against bacteria, fungi, plants and tumour cell lines, and inhibits aromatase and sulfatases. Questioniomycin, like other phenoxazines, stimulates cell growth and turnover *in vitro*, an activity possibly related to their ability to form stable free radicals (Anzai et al., 1960). More recently, questioniomycin A has been shown to inhibit pulmonary metastasis caused by mouse melanoma cells (Hongo et al., 2010).

The colony size of *P. expansum* on crème fraiche was quite small, around 1 cm², and could be ignored as unimportant or even not detected by a consumer. Despite this, *P. expansum* produced high concentrations of patulin in crème fraiche (1440 ± 237 µg/kg in layer A), albeit at lower concentrations than in apple jam in the previous study (34,070 ± 3690 µg/kg in layer A; Olsen et al., 2017). However, in crème fraiche this isolate of *P. expansum* (M17) produced a high concentration of citrinin, 1880 ± 662 µg/kg in layer A. This isolate (M17) is capable of producing citrinin on YES-agar according to the previous study but citrinin was not produced in the fruit jams (apple and blueberry) in that study.

The concentration of patulin in top layer (A) of the crème fraiche would correspond to an exposure above the TDI of 0.4 µg/kg b.w. per day (JECFA, 1995) for a normal serving of crème fraiche (50 g) to an

Table 2

Mycotoxins and some other major secondary metabolites at different distances from fungal colony on the surface of apple jam or crème fraiche after 14 days incubation at 15 °C.

Fungal species (colony diameter) ^a	Secondary metabolite	Distance from mouldy surface		
		0–2 cm ^b	2–4 cm	> 4 cm
(µg/kg ± s.d.) ^c				
<i>Apple jam</i>				
<i>Penicillium crustosum</i> (41 ± 0.7)	Cyclophenol	3460 ± 663	3.4 ± 0.3	15.3 ± 21.7
	Penitrem A	7.6 ± 1.0	< LOD ^d	< LOD
	Roquefortine C	12,500 ± 1980	< LOD	< LOD
<i>Penicillium roqueforti</i> (55 ± 0.7)	Andrastin A	3030 ± 340	1.1 ± 0.3	1.2 ± 1.7
	Festucaclavine	1570 ± 290	10.4 ± 14.3	< LOD
	Fumigaclavine A	2020 ± 238	5.5 ± 5.8	0.1 ± 0.1
	Mycophenolic acid	416 ± 46	2.11 ± 3.0	< LOD
	Roquefortine C	8510 ± 968	< LOD	< LOD
	Questioniomycin A	180 ± 64	25 ± 9.1	0.6 ± 0.8
<i>Light crème fraiche</i>				
<i>Penicillium expansum</i> (12 ± 2.5)	Citrinin	1880 ± 662	0.2 ± 0.3	< LOD
	Patulin	1440 ± 237	322 ± 115	< LOD

^a Colony diameter expressed as mm ± standard deviation (n = 2).

^b Includes the fungal colony.

^c Concentration in each layer expressed as µg/kg ± standard deviation (n = 2).

^d < LOD indicates that levels are less than limit of detection. There were no results > LOD but < LOQ.

adult (70 kg b.w.). The concentration in layer B would possibly exceed the TDI at a high consumption rate (100 g).

EFSA's CONTAM Panel (2012) concluded that due to the limitations and uncertainties in the database, the derivation of a health-based guidance value for citrinin was not considered appropriate but a level of no concern for nephrotoxicity of 0.2 µg/kg b.w. per day was determined (EFSA, 2012). A normal serving of crème fraiche (50 g) from layer A would correspond to more than six times the guidance value. On the other hand, citrinin was not as widely distributed as patulin throughout the crème fraiche and the concentration in layer B would not indicate a risk for exposure above the guidance value.

Apart from patulin and citrinin, none of the biologically active substances included in Table 2 has a HBGV.

3.2. Growth and mycotoxin production of *P. expansum* in apple jam

Growth of *P. expansum* was observed on the surface (top layer) of the apple jam at all storage temperatures (Fig. 1a). Growth rates estimated by fitting to the Baranyi model increased with storage temperatures and were 1.7, 2.7 and 4.3 mm day⁻¹ (growth rates) for storage at 4, 8 and 15 °C, respectively. In contrast, apparent lag times decreased with increasing storage temperatures and were 10.6, 7.9 and 2.6 (days) at the corresponding temperatures. Simple linear secondary predictive models for growth rates and lag times of *P. expansum* in apple jam were estimated in this temperature range (Fig. 1b).

The major fungal metabolites of *P. expansum* identified and quantified in apple jam were patulin, roquefortine C, andrastin A and communesin B. All those were identified in layer A in apple jam incubated at 15 °C (Fig. 2). Both andrastins and communesins have been used in drug development in antitumor research programs. Andrastin A inhibits the farnesyltransferase activity of the oncogenic Ras proteins, and also promotes the intracellular accumulation of anticancer compounds in tumoral cells (Rojas-Aedo et al., 2017). Communesin B, on the other hand, is a cytotoxic alkaloid tested positively for anti-proliferative activity against several leukemia cell lines (Trost and Osipov, 2015). Andrastins are also naturally occurring in blue cheese together with other fungal metabolites such as the mycotoxin roquefortine C (Fernández-Bodega et al., 2009). Andrastins can be produced both by *P. expansum* and *P. roqueforti*. The other fungal metabolites, patulin and roquefortine C, are regarded as mycotoxins (Bråse et al., 2009).

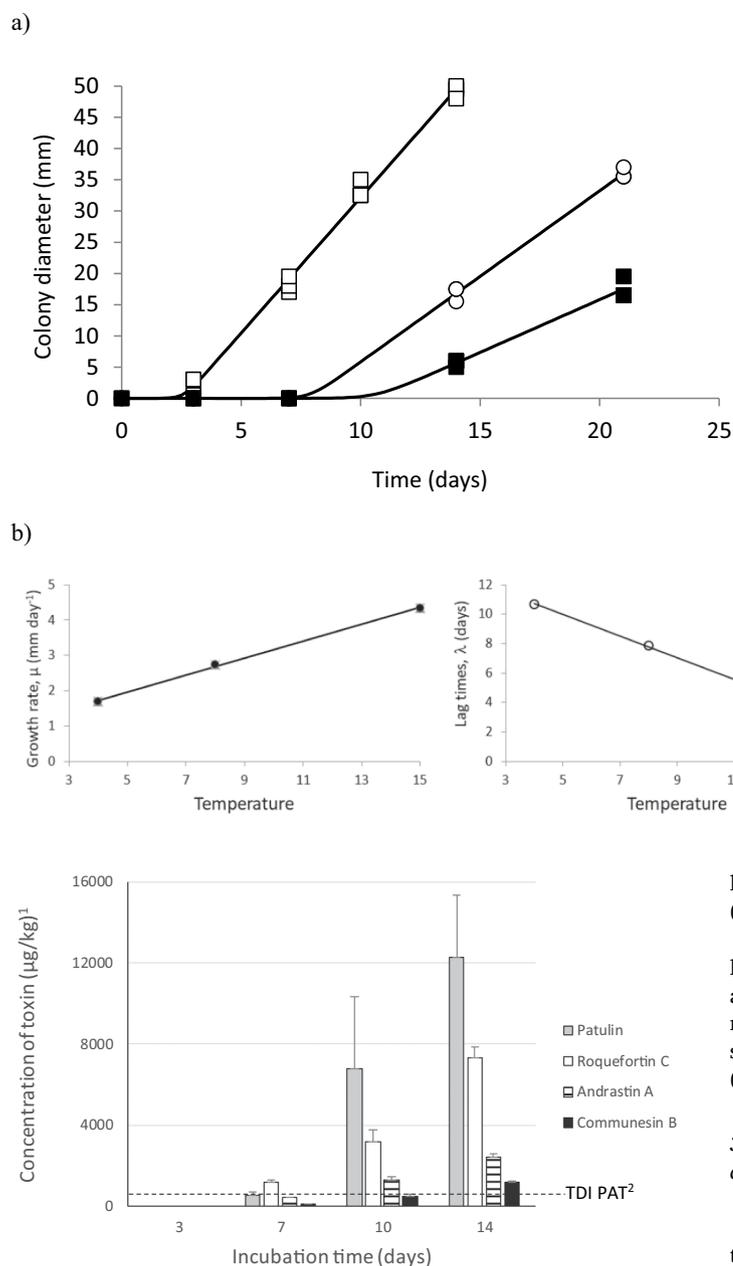


Fig. 2. Major fungal metabolites produced in top layer A of apple jam during incubation at 15 °C.

1. Concentration in layer A (0–2 cm parallel with the top surface including the fungal colony), $n = 3$.
2. Concentrations above the dotted line, 549 $\mu\text{g}/\text{kg}$, correspond to an intake above TDI for patulin at median portion size in an adult person (body weight: 70 kg).

The dotted line in Fig. 2 indicates a concentration of patulin (549 $\mu\text{g}/\text{kg}$), which corresponds to the TDI for patulin, 0.4 $\mu\text{g}/\text{kg}$ bodyweight (JECFA, 1995), at a median portion size (51 g) for apple jam consumers (NFA, 2012) in an adult person of 70 kg b.w. The concentration in layer B (data not shown in Fig. 2) after 14 days (2490 \pm 680 $\mu\text{g}/\text{kg}$) was more than twice as much as the TDI; consequently, removal of only the top layer A would not be sufficient to reduce the patulin concentration to a tolerable level. There is no HBGV established for roquefortine C, andrastin A or communesin B.

Analyses of apple jam samples incubated at 15 °C were stopped after 14 days since the colony had reached the edge of the jar. Patulin was not detected in any samples incubated at 4 °C, and at 8 °C only in the top

Fig. 1. Observed growth of *P. expansum* in apple jam stored at different temperatures ($n = 3$) and fitted growth rates and lag times of a) primary and b) secondary models, respectively.

a) Observed colony diameter in apple jam stored at 4 °C (■), 8 °C (○), and 15 °C (□). The lines describe the biphasic Baranyi growth model fitted to the data. b) The lines describe secondary predictive models for *P. expansum* growth rate (mm day⁻¹) and apparent lag time (days) in apple jam. Growth rates (μ , ●) and lag times (λ , ○) estimated by fitting observed growth to the biphasic Baranyi model at the different storage temperatures (see a) were fitted to linear models: growth rate = 0.240 * temperature + 0.768, lag time = 13.653 – 0.735 * temperature. The adjusted R-squared was > 0.99 for both lines.

layer A. In contrast, roquefortine C was produced at all temperatures (Fig. 3) but was not detected in layer B and C except at 15 °C.

When the total amount of toxin in apple jam, sum of toxin in all layers, was plotted as a function of the *P. expansum* colony diameter, it appeared that roquefortine C production started when colony diameters reached 15–20 mm at all temperatures examined, whereas the same sized colonies produced patulin only at 15 °C and not at 8 or 4 °C (Fig. 4).

3.3. Growth of *P. verrucosum*, production and distribution of mycotoxins in crème fraîche depending on temperature and time

The growth of *P. verrucosum* in crème fraîche was very restricted, the surface of the colony was wrinkled, the colour was pinkish, and the general appearance was atypical for this species (Fig. 5). One hypothesis is that the restricted and wrinkled appearance was due to the competition from lactic acid bacteria in the crème fraîche, which was very fresh and had just been delivered from the dairy. The increase in colony diameter in relation to time and temperature at 15 °C is shown in Fig. 6. The growth of *P. verrucosum* at 4 °C was only observed after 28 days (6.3 \pm 2.8 mm) although then at the same size as for 8 °C (5.8 \pm 1.4 mm). These results suggest that the experiments should be repeated using crème fraîche which is not freshly taken from the dairy, but rather, near the end of the “best before date”.

Formation of ochratoxin A and citrinin was observed after 21 days of incubation and only in subsample A at 15 °C (Fig. 6) except for one of the triplicate samples from 28 days at 8 °C which contained 1.8 μg ochratoxin A/kg. However, the concentration of ochratoxin A at 21 and 28 days is relatively high despite the small colony size (Fig. 6). The dashed line for the TDI of ochratoxin A indicates the concentration above which a normal recipe serving, containing 50 g crème fraîche per portion, results in an exposure above TDI (EFSA, 2006) for an adult person (70 kg b.w.). So just removing the small colony from the surface of the crème fraîche would most likely leave a substantial amount of ochratoxin A in the product. Both ochratoxin A and citrinin are heat

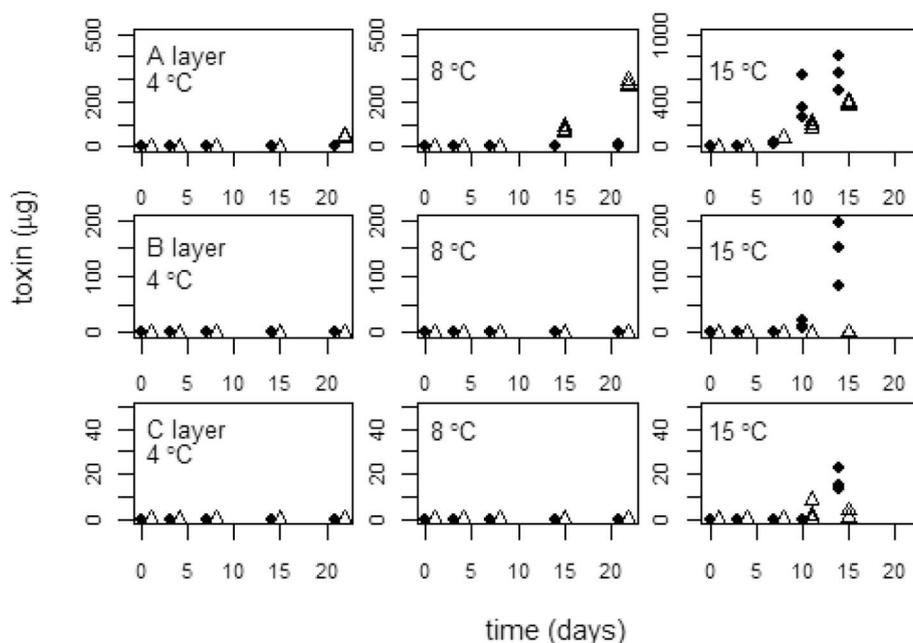


Fig. 3. The total amount of toxin detected in the top (A), middle (B) and bottom (C) layers of apple jam stored at different temperatures. Patulin (●) and roquefortine C (Δ). Note the different scales on the y-axes in the different layers and that roquefortine C is plotted on the day after analysis, i.e. time + 1, to improve visibility.

stable and recipes including crème fraiche may be without heating or just a short time of cooking. According to Boudra et al. (1995), 150 °C is needed to degrade ochratoxin A and 160 °C for citrinin (Trivedi et al., 1992) and consequently none or no major degradation of ochratoxin A and citrinin is expected during cooking. Ochratoxin A is nephrotoxic and classified as a type 2B carcinogen (IARC, 1993); sources of exposure are many, including cereals and cereal products, pulses, coffee, beer, grape juice, dried vine fruits and wine, as well as cacao products, nuts and spices. Furthermore, contamination of animal feeds with ochratoxin A may result in the presence of residues in edible offal and

blood serum (EFSA, 2006). In other words, it is important to keep exposure to ochratoxin A from known sources as low as possible and not add residues from mouldy food.

As mentioned earlier, there is no tolerable intake established for citrinin but instead a guidance value. The dotted line of citrinin indicates the concentration above which a normal recipe serving, containing 50 g crème fraiche per portion, results in an exposure above the HBGV for an adult person (70kg b.w.). Similar to ochratoxin A, citrinin is nephrotoxic. In a recent study (Rašić et al., 2018), the combined effect of ochratoxin A and citrinin was studied in Wistar rats on

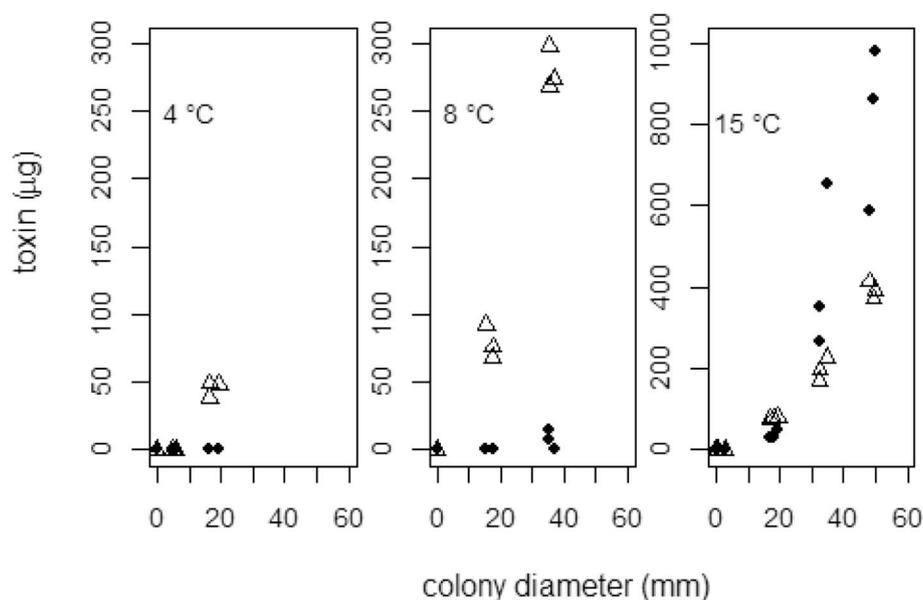


Fig. 4. The total amount of toxin, i.e. the sum of toxin detected in the top, middle and bottom layers, depending on the colony diameter of apple jam stored at different temperatures.

Patulin (●) and roquefortine C (Δ). Note the different scales on the y-axes at the different temperatures.



Fig. 5. Photo of *P. verrucosum* colony on crème fraîche after 21 days at 15 °C.

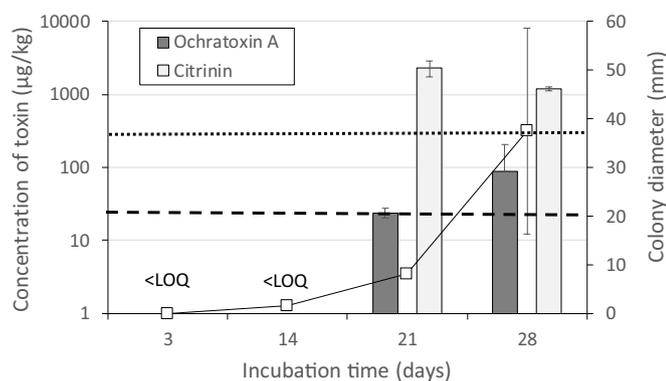


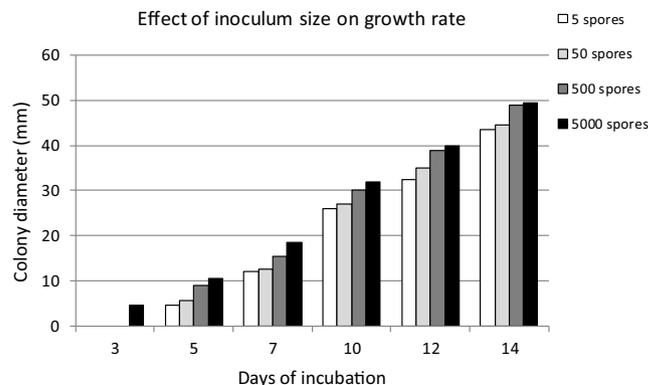
Fig. 6. Growth and production of ochratoxin A and citrinin by *P. verrucosum* in 2 cm-top layer of crème fraîche at 15 °C.

Concentrations above the dotted line, 280 µg/kg, in a median portion size containing crème fraîche (50 g) are above the health based guidance value for citrinin in an adult person (body weight: 70 kg). Concentrations above the dashed line, 23.8 µg/kg, in a median portion size containing crème fraîche (50 g) are above the TDI for ochratoxin A in an adult person (body weight: 70 kg). Observed colony diameter of *P. verrucosum* on crème fraîche stored at different temperatures (□). N = 3 except at 28 days due to one missed inoculation (n = 2).

oxidative damage to kidneys and liver. Most effects in the combined treatments were attributed to the effects of ochratoxin A.

Besides the two mycotoxins ochratoxin A and citrinin, *P. verrucosum* produced another major metabolite in crème fraîche, questiomycin A, the biological effects of which have been described above. The concentration of questiomycin A in layer A at 15 °C was 136 ± 35 µg/kg (n = 3) and 341 ± 447 µg/kg (n = 2) after 21 and 28 days, respectively. In contrast to ochratoxin A and citrinin, questiomycin A was detected both in layers B and C, reaching 26 ± 6.0 and 91 ± 112 µg/kg in layer B after 21 and 28 days, respectively, and 4.2 ± 2.3 and 26 ± 29 µg/kg in layer C after 21 and 28 days, respectively. Lower concentrations of questiomycin A (< 35 µg/kg) in layer A were also detected in samples incubated at 4 and 8 °C. There is no HBGV established for questiomycin and therefore it is not possible to assess the risk of the detected concentrations.

A



B

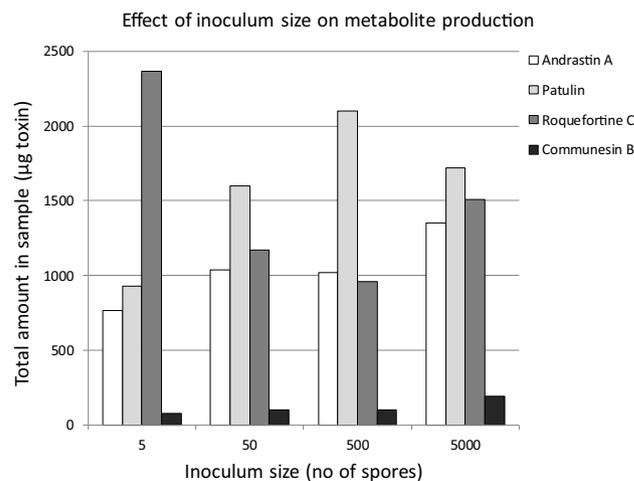


Fig. 7. The effect of inoculation size of *P. expansum* on growth rate (A) and metabolite production in apple jam after 14 days (B).

3.4. Effect of the inoculation size on growth and production of fungal metabolites

The inoculation size used generally in this study, 2500 spores/inoculation, is the one that we used in the previous study (Olsen et al., 2017). The results of the inoculation size study are shown in Fig. 7 A + B. Not unexpectedly, colony size over time appeared to be dependent on inoculation size (Fig. 7A), but colony diameter growth rates estimated by fitting to the Baranyi model were the same, 4.2–4.5 mm day⁻¹. The estimated apparent lag time decreased with increasing inoculum size, and was 4.1, 3.9, 3.3 and 2.3 days for inoculum sizes of 5, 50, 500 and 5000, respectively. In contrast to growth, the metabolite pattern and concentrations (Fig. 7B) did not follow the same clear trends. For example, the highest concentration of roquefortine C was associated with the lowest inoculation size. This is only a single pilot study and consequently we cannot draw any conclusions, but the results indicate that these effects of inoculation size on toxin production should be further investigated, as this may have implications for the best inoculum size to use in experimental studies. Further extrapolating the potential impact of low inoculum size to the real-life scenario of colonies originating from single spores is also of interest.

4. Conclusions

- Substantial amounts of biologically active fungal metabolites are formed when food moulds. Of these substances, only a few are toxicologically characterized to the extent of being given a HBGV.

- The production and distribution of fungal toxins in a food seems to depend on several factors including fungal species/strain, storage temperature, size of toxin molecules, and the properties of the food such as liquidness.
- Mould growth and toxin production is delayed when food is stored at low temperatures, but when mould growth becomes visible (a common definition of visible mould growth is > 3 mm diameter), substantial amounts of mycotoxins in ranges of toxicological concern may be formed, despite a small colony size. Formulation of general advice for consumers is, thus, difficult, except to avoid mouldy food.

Declaration of interest

None.

Acknowledgements

This study was financially supported by University of Natural Resources and Life Sciences, Vienna, Austria, the Swedish National Food Agency and the Swedish University of Agricultural Sciences (SLU), Faculty of Natural Resources and Agricultural Sciences. The authors wish to thank Ann Gidlund for technical assistance.

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