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Protection by fungal starters against growth and secondary metabolite production of fungal spoilers of cheese

Marianne S. Nielsen*, Jens C. Frisvad, Per V. Nielsen

Department of Biotechnology, Building 221, Technical University of Denmark, DK-2800 Lyngby, Denmark

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Abstract

The influence of fungal starter cultures on growth and secondary metabolite production of fungal contaminants associated with cheese was studied on laboratory media and Camembert cheese. Isolates of the species *Penicillium nalgiovense*, *P. camemberti*, *P. roqueforti* and *Geotrichum candidum* were used as fungal starters. The species *P. commune*, *P. caseifulvum*, *P. verrucosum*, *P. discolor*, *P. solitum*, *P. coprophilum* and *Aspergillus versicolor* were selected as contaminants.

The fungal starters showed different competitive ability on laboratory media and Camembert cheese. The presence of the *Penicillium* species, especially *P. nalgiovense*, showed an inhibitory effect on the growth of the fungal contaminants on laboratory media. *G. candidum* caused a significant inhibition of the fungal contaminants on Camembert cheese. The results indicate that *G. candidum* plays an important role in competition with undesirable microorganisms in mould fermented cheeses.

Among the starters, *P. nalgiovense* caused the largest reduction in secondary metabolite production of the fungal contaminants on the laboratory medium. On Camembert cheese no significant changes in metabolite production of the fungal contaminants was observed in the presence of the starters.

Keywords: Cheese; Fungi; *Geotrichum candidum*; Inhibition; *Penicillium* spp.; Secondary metabolites

1. Introduction

Some dairy products, especially cheese, are favourable substrates for fungal growth (Lund et al., 1995; Northolt et al., 1995). The growth of fungal contaminants on cheese may produce off-flavours, mycotoxins or discoloration of the surface (Marth and Yousef, 1991). Previous studies concerning mycotoxin production on cheese have mainly been carried out with aflatoxin-producing aspergilli in spite of the rare occurrence of *A. flavus* on cheese (Scott, 1989; Marth and Yousef, 1991). *Penicillium* species are the most common species found in cheese and it is mainly their toxins which have been identified in cheese. These include roquefortin C, isofumigaclavine A (Scott et al., 1976), cyclopiazonic acid (Le Bars, 1979), mycophenolic acid (Lafont et al., 1979), ochratoxin A (Jarvis, 1983) and PR toxin (Siemens and Zawistowski,

*Corresponding author. Tel.: +45 45252727; fax: +45 45884922; e-mail: msn@ibt.dtu.dk

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1993). Besides aflatoxins the *Aspergillus* mycotoxin sterigmatocystin has been found to be produced naturally in cheese (Northolt et al., 1980).

Several species of fungi are used to manufacture mould ripened foods. In Europe, these foods are primarily cheeses and meats, where *Penicillium* species in particular are used as starter cultures. In addition, *Geotrichum candidum* is a natural part of the surface funga of several varieties of cheese, including Camembert (Rousseau, 1984; Marth and Yousef, 1991).

Starter cultures contribute to the inhibition of the undesirable growth of fungal contaminants and mycotoxin production in fermented food. There have been very few fungal interaction studies in fermented dairy products such as cheese. *G. candidum* has been found to be affected by the presence of *Brevibacterium linens* depending on pH, salt and temperature (Lecocq and Gueguen, 1994) and affected by the presence of *P. camemberti* on experimental Camembert cheese (Molimard et al., 1995). In another mould fermented product, dry sausage, Berwal and Dincho (1995) found that the use of *P. camemberti* and *P. nalgiovense*, sprayed as a conidial suspension with 10^8 spores per ml on sausages, completely eliminated the growth of undesirable moulds originating from the natural house funga.

The aim of this work was to examine how growth and secondary metabolite production by fungal contaminants in cheese is influenced by the presence of fungal starter cultures.

2. Materials and methods

2.1. Media

The experiments were carried out on two laboratory media. Czapek yeast extract agar (CYA) was used as the standard medium for fungi (Samson et al., 1995). In addition, a cheese agar medium developed especially for the growth of cheese related fungi was used (Hansen and Nielsen, 1996). The cheese agar contained: 100 g casein, 8.3 g lactate (90%), 7.9 g lactose, 7.3 g $CaCl_2 \cdot 2H_2O$, 2.6 g $MgSO_4 \cdot 7H_2O$, 26.0 g NaCl, 20 g agar, 0.025 g $FeSO_4 \cdot 7H_2O$, 0.004 g $CuSO_4 \cdot 5H_2O$, 250 g unsalted butter and water to a total weight of 1 kg. The casein, agar, butter and $CaCl_2 \cdot 2H_2O$ (total weight 600 g) were autoclaved separately to avoid acid hydrolysis of the agar and Maillard reaction. Lactate, lactose, $MgSO_4 \cdot 7H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 5H_2O$ (total weight 400 g) were autoclaved simultaneously in a separate bottle in which the pH was adjusted to 5.5 with 10 M NaOH before the solution was autoclaved. After cooling to 50°C, the content of the two bottles was combined, and the medium was poured into 9 cm Petri dishes.

2.2. Camembert cheese

Besides the laboratory media, Camembert cheese was used. The cheeses were used for experiments just before ripening (at 17–18°C in 6 days). At this stage the cheeses have been salted, and starter cultures and rennet have been added. Each cheese was cut into three pieces with a sterile knife and placed in 9 cm Petri dishes.

2.3. Fungal cultures

Fungal cultures isolated from cheese were obtained from the Fungal Culture Collection at the Department of Biotechnology (IBT), Technical University of Denmark. Conidia were obtained from 10 day old colonies grown on Czapek yeast extract agar (CYA) at 25°C and suspended in a semi-solid medium containing 0.5% agar and 0.5% Tween 80 and diluted to a final concentration of 10^6–10^7 spores ml^{-1}. This concentration is the recommended concentration of fungal starter culture added just after salting in the production of white mould cheese (Philipp and Pedersen, 1988). *Geotrichum candidum* IBT 97877, *Penicillium camemberti* IBT 912012, *P. nalgiovense* IBT 3793 and *P. roqueforti* IBT 4176 were selected as starter cultures. Concerning the contaminants, the experiment was conducted in two parts.

Experiment 1: *P. commune* IBT 12080, *P. caseifulvum* IBT 14437, *P. verrucosum* IBT 13045, *P. discolor* IBT 13777 and *A. versicolor* IBT 12384 were used as fungal contaminants.

Experiment 2: *P. commune* IBT 18286, *P. caseifulvum* IBT 18282, *P. verrucosum* IBT 6734, *P. discolor* IBT 12098, *A. versicolor* IBT 12599, *P. solitum* IBT 12595 and *P. coprophilum* IBT 3063 were used as fungal contaminants. *P. coprophilum* was the only contaminant not isolated from cheese.

This species was isolated from sausage and was used to compare cheese-associated species with a species from another habitat.

2.4. Inoculation

The conidial suspension (0.1 ml) of each fungal starter was surface spread on CYA and cheese agar in experiment 1, and on CYA, cheese agar and Camembert cheese in experiment 2. Then 0.01 ml of the conidium suspensions of the fungal contaminants were inoculated at three points onto plates with starters. Inoculation of the contaminants on pure media was applied as a control. All the combinations were incubated at 18 and 25°C for 7 days in darkness. All experiments were performed in duplicate. The diameter of the contaminants was measured and cultural extracts were prepared as described below.

2.5. Extraction procedure

The extraction procedure was modified from Smedsgaard (1997). Cultural extracts were prepared by cutting a plug (6 mm) of agar in the centre of four contaminant cultures using a cork borer. The plugs were transferred to 1.5 ml autosampler screw-cap vials and 400 μl dichloromethane/methanol/ethyl acetate (2:1:3) with 0.5% (v/v) formic acid was added. The vials were placed in an ultrasonic bath (Branson 3200, Holland) for 30 min. The liquid was filtered through a phase separation filter (Whatman No. 1) into a 15 ml preparation glass. The organic phase was evaporated in a stream of nitrogen and redissolved ultrasonically for 10 min in 400 μl methanol. The final extract was passed through a 0.45 μm filter.

2.6. HPLC procedure

The HPLC procedure was modified from Frisvad and Thrane (1987) and Smedsgaard (1997). The HPLC analyses were performed on a Hewlett-Packard HP 1090M HPLC by injecting 10 μl into a 100 mm × 4 mm HP Hypersil BDS-C18 Cartridge column with a 4 mm × 4 mm guard cartridge. The column was held at 40°C. A solvent gradient with water and acetonitrile (with 0.05% trifluoroacetic acid added) was used, starting with 85% water and 15% acetonitrile, and raised to 100% acetonitrile within 40 min and then maintained at 100% acetonitrile for 3 min. Detection was made by a photodiode array (PDA) detector with a 6 mm flow cell at a wavelength of 225 nm. A PDA detector recorded the UV spectra in the range 200–600 nm.

2.7. Data analysis

Data from measurements of growth and metabolite production were analysed statistically using partial least squares regression (PLS) (Wold et al., 1984) in SIMCA-P for Windows (Version 2.1, 1995, Umetri AB, Sweden). PLS is a multivariate method with the same theoretical foundation as principal component analysis (PCA). In projection methods such as PCA and PLS the relevant information in a data matrix (table of experimental data) is determined by projecting the data to a space with a lower dimension. In PLS, two matrices, X and Y, are related to each other so that the information in Y has an influence on the bilinear decomposition of X into a low number of components (PLS components). The significant PLS components found by cross-validation are used to evaluate the statistical model (Martens and Næs, 1989; Höskuldsson, 1996). A PLS component explains a certain percentage of the variation in the data (R^2). The sum of R^2 values for the significant components above 80% in the PLS analysis indicates an acceptable regression model. In this work the x-variables (factors) consist of the presence of fungal starters (binary data) and the temperature (quantitative data). The y-variables (responses) in the growth data are the colony diameter of the fungal contaminants and in the metabolite data the number of peaks detected and the area of peaks (mAU · sec) detected. The data are presented as loading plots that show the relationships between the x- and y-variables and the importance of each variable.

3. Results and discussion

3.1. Growth of fungal contaminants

Analysis of the data from the radial growth of the fungal contaminants using PLS and cross-validation indicated three significant components when each substrate was analysed separately. The variance explained by each component is shown in Table 1.

Table 1
Variance explained on the significant components (% cumulative) for colony diameter in the PLS model

Response variables	Component 1	Component 2	Component 3
Colony diameter on CYA (experiments 1 and 2)	84	92	94
Colony diameter on cheese agar (experiments 1 and 2)	72	86	91
Colony diameter on Camembert cheese (experiment 2)	63	67	71

Using the data from CYA, cheese agar and Camembert cheese a model with $R^2 = 94$, 91 and 71%, respectively, was obtained. For the data from all three media most of the variation was explained by component 1 (84, 72 and 63%, respectively). Figs. 1 and 2 show loading plots for components 1 and 2 from measurements on CYA and Camembert cheese, respectively. Factors located furthest from the origin have the largest influence on the responses. The plots show that the presence of the fungal starters describes the variation obtained by the first component. All the fungal contaminants are affected in the same way by being inhibited in growth rate when fungal starters are present as compared to control media without a starter culture. The presence of the *Penicillium* starters, in particular *P. nalgiovense*, was the most important factor for CYA (Fig. 1) and cheese agar. The presence of *G. candidum* explained the variation obtained by the second component and separated the contaminants. *P. verrucosum*, *P. commune* and *P. caseifulvum* which were most inhibited by *G. candidum*. On Camembert cheese, the presence of *G. candidum* was the most important factor describing almost all the variance explained by component 1 (Fig. 2). The presence of the *Penicillum* starter on Camembert cheese did not show any effect on the contaminants.

The two isolates of the contaminants *P. commune*, *P. verrucusom*, *P. caseifulvum*, *P. discolor* and *A. versicolor* were close to each other in the loading plot from inoculation on CYA (Fig. 1) and cheese agar showing that isolates of the same species were affected in the same way by the parameters. *P. coprophilum* was the only species not associated with cheese. This fungus is normally associated with dung. This species is affected in the same way by the fungal starters as the cheese-related species (Figs. 1 and 2).

The reduction of the radial growth of fungal contaminants by the presence of *G. candidum* and *P. nalgiovense* is shown in Tables 2 and 3, respectively. These results show that the difference in inhibition of the fungal contaminants by *P. nalgiovense* on different media is most pronounced at 18°C. At this temperature the fungal contaminants were, on aver-

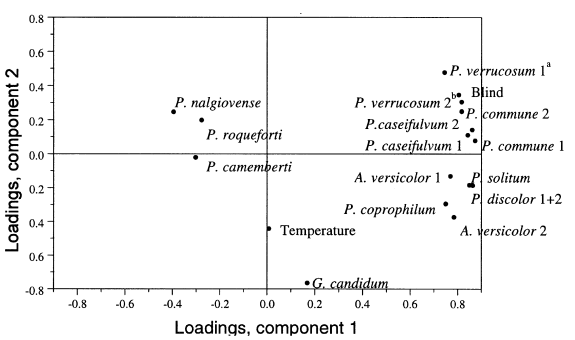

Fig. 1. PLS loading plot showing the relationship between X = presence of the fungal starters (*G. candidum*, *P. camemberti*, *P. nalgiovense* and *P. roqueforti*) and temperature, and Y = radial growth of fungal contaminants (*P. commune*, *P. caseifulvum*, *P. verrucosum*, *P. discolor*, *P. solitum*, *P. coprophilum* and *A. versicolor*) on CYA after 7 days incubation. [a]Isolates denoted 1 correspond to experiment 1. [b]Isolates denoted 2 correspond to experiment 2.

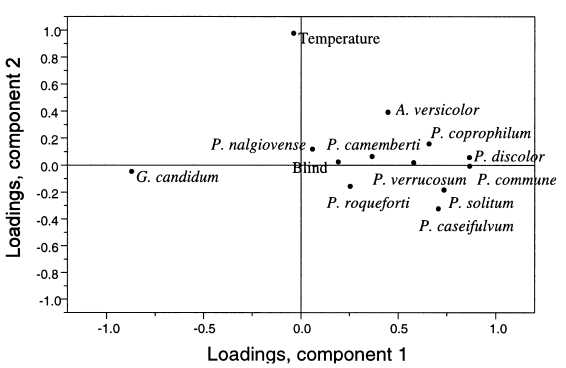

Fig. 2. PLS loading plot showing the relationship between X = presence of the fungal starters (*G. candidum*, *P. camemberti*, *P. nalgiovense* and *P. roqueforti*) and temperature, and Y = radial growth of the fungal contaminants (*P. commune*, *P. caseifulvum*, *P. verrucosum*, *P. discolor*, *P. solitum*, *P. coprophilum* and *A. versicolor*) on Camembert cheese after 7 days incubation.

Table 2
Reduction of radial growth of fungal contaminants (%) in the presence of *G. candidum* compared to medium without fungal starter after 7 days incubation

Contaminant	CYA		Cheese agar		Camembert cheese	
	18°C	25°C	18°C	25°C	18°C	25°C
P. commune 1[a]	45	28	0	8	–[d]	–[d]
P. commune 2[b]	37	66	20	13	100	100
P. caseifulvum 1	32	27	12	24	–[d]	–[d]
P. caseifulvum 2	31	46	17	13	27	100
P. verrucosum 1	54	51	0	36	–[d]	–[d]
P. verrucosum 2	48	49	0	15	–[c]	100
P. discolor 1	24	10	4	17	–[d]	–[d]
P. discolor 2	16	21	12	25	100	100
A. versicolor 1	13	29	0	18	–[d]	–[d]
A. versicolor 2	5	9	0	7	–[c]	100
P. solitum	21	28	17	31	100	100
P. coprophilum	0	26	6	31	3	100
Average	27	33	7	20	66	100

[a]Isolates denoted 1 correspond to experiment 1.
[b]Isolates denoted 2 correspond to experiment 2.
[c]No detection of fungal growth on media without fungal starters (blind).
[d]Not measured.

age, reduced 57% on CYA and 50% on cheese agar by *P. nalgiovense* compared to 5% on Camembert cheese. At 25°C the presence of *P. nalgiovense* reduced the radial growth of the contaminants by 51–67%, on average, on the three media. The presence of *G. candidum* only reduced the contaminants up to 33%, on average, on the laboratory media compared to 66 and 100% on Camembert cheese at 18 and 25°C, respectively.

The different competitive abilities of the fungal

Table 3
Reduction of radial growth of fungal contaminants (%) in the presence of *P. nalgiovense* compared to media without fungal starter after 7 days incubation

Contaminant	CYA		Cheese agar		Camembert cheese	
	18°C	25°C	18°C	25°C	18°C	25°C
P. commune 1[a]	65	69	50	65	–[d]	–[d]
P. commune 2[b]	58	69	56	61	6	24
P. caseifulvum 1	54	51	51	59	–[d]	–[d]
P. caseifulvum 2	61	58	54	61	17	0
P. verrucosum 1	47	56	55	65	–[d]	–[d]
P. verrucosum 2	57	49	70	67	–[c]	100
P. discolor 1	60	66	52	78	–[d]	–[d]
P. discolor 2	63	64	42	60	0	32
A. versicolor 1	46	56	33	58	–[d]	–[d]
A. versicolor 2	45	66	21	62	–[c]	100
P. solitum	63	66	48	69	0	100
P. coprophilum	62	55	58	100	0	0
Average	57	60	50	67	5	51

[a]Isolates denoted 1 correspond to experiment 1.
[b]Isolates denoted 2 correspond to experiment 2.
[c]No detection of fungal growth on media without fungal starters (blind).
[d]Not measured.

starters on laboratory media and Camembert cheese observed in this study may be due to different intrinsic factors. Physiological studies of fungal growth parameters such as the concentration of NaCl and pH have shown significant effects on growth of the four fungal starter species used in this study (Choisy et al., 1987a,b; Desmazeaud and Cogan, 1996; Haasum and Nielsen, 1997). Morris (1964) and Godinho and Fox (1981) observed that growth of *P. roqueforti* is frequently absent or poor in the outer regions of blue cheese due to localized high salt concentrations. Choisy et al. (1987a) found that a relatively low salt level limits the growth of *G. candidum* with no notable effect on *P. camemberti*. *P. nalgiovense* is characterized as having a high NaCl tolerance (Philipp and Pedersen, 1988).

The media, CYA and cheese agar, used in this study contain 0.05 and 4.5% NaCl and have a pH of 6.5 and 5.5, respectively. Camembert cheese just after salting contains 4% NaCl on the surface (Hardy, 1987) and the pH is about 4.5 on the surface (Lenoir, 1984; Chapman and Sharpe, 1990). These values were used in a PLS analysis including all three media (data not shown). The NaCl concentration and pH explained most of the variation obtained by component 1, but the model was not significant ($R^2 = 47\%$). This indicates that the observed differences between the use of the laboratory media and the Camembert cheese are not fully explained by differences in NaCl concentration and pH.

Many parameters other than salt and pH were different between the laboratory media and Camembert cheese (water content, structure, content of microorganisms, etc.). Studies of the microbial growth of starters in the production of Camembert cheese have shown that *G. candidum* has good growth conditions on Camembert cheese just after salting. Species of *Geotrichum* appear on the cheese surface 2–3 days after salting. A few days later *P. camemberti* begins to develop and spreads over the whole surface (Rousseau, 1984; Chapman and Sharpe, 1990). From this description it appears reasonable that *G. candidum* is a good competitor on Camembert cheese.

G. candidum is normally present in mould fermented cheese, in white (Nooitgedagt and Hartog, 1988) as well as blue cheeses (López-Diaz et al., 1995). Excessive growth of *G. candidum* in Camembert will result in a slimy cheese surface and inhibit growth of *P. camemberti*. In reasonable quantities the presence of *G. candidum* is desirable because it improves the organoleptic qualities of the cheese (Choisy et al., 1987b). The results obtained in this study indicate that this fungal species also plays an important role in competition with undesirable contaminants in the cheese.

3.2. Metabolite production of fungal contaminants

HPLC analysis of extracts from fungal contaminants grown on CYA and Camembert cheese showed from 0 to 14 peaks and from 0 to 3 peaks, respectively. Several known metabolites were identified using retention time and UV spectra by comparison to analyses of standards. The results described below are from inoculation on CYA and Camembert cheese. No metabolites were detected from inoculation on cheese agar in experiment 1. Therefore, no further analyses of this medium were made.

HPLC analysis of extracts of CYA inoculated with fungal starters gave 0–2 peaks and no known mycotoxins were detected; however, between 0 and 14 peaks were detected from the fungal contaminants. Among these were cyclopiazonic acid from both isolates of *P. commune*, sterigmatocystin from both isolates of *A. versicolor*, chaetoglobosin A from both isolates of *P. discolor*, ochratoxin A from one of the isolates of *P. verrucosum*, viridicatin and cyclopenol from *P. solitum* and griseofulvin from *P. coprophilum* (Table 4).

The presence of *P. nalgiovense* was the most important factor in the PLS analysis for the detection of the above-mentioned mycotoxins expressed as peak areas ($R^2 = 70\%$). This fungus explained most of the variance obtained by component 1, thereby showing the largest inhibition of the production of mycotoxins among the fungal starter. The most significant model ($R^2 = 87\%$) was obtained by analysis of the mycotoxin detected in largest quantities, griseofulvin (Table 4).

Data with the total number of metabolites detected from each contaminant (metabolites detected from the starters were excluded) were analysed by PLS. It was difficult to obtain a good model including all the contaminant isolates ($R^2 = 67\%$). For some of the isolates very few metabolites were detected (less than four peaks). Excluding these isolates a good

Table 4
Peak area (mAU · sec) of mycotoxins produced by fungal contaminants grown on CYA or Camembert cheese in 7 days

Starter	Temp. (°C)	Medium											
		CYA		CYA		CYA		CYA		CYA	CYA	CYA	Cheese
		Cyclopiazonic acid *P. commune*		Chaetoglobosin A *P. discolor*		Sterigmatocystin *A. versicolor*		Ochratoxin A *P. verrucosum*		Cyclopenol *P. solitum*	Viridicatin *P. solitum*	Griseofulvin *P. coprophilum*	
		1[a]	2[b]	1	2	1	2	1	2	2	2	2	2
G. candidum	18	nd	236	5552	1232	nd	nd	nd	421	nd	238	5282	563
P. camemberti	18	488	1239	8485	2828	206	448	nd	nd	1219	318	6236	645
P. nalgiovense	18	nd	352	1963	1021	nd	nd	nd	nd	nd	nd	2267	397
P. roqueforti	18	248	335	4679	3069	nd	nd	nd	nd	1080	nd	5443	114
Blind	18	2281	1300	6380	1790	218	1232	nd	403	1580	531	23462	366
G. candidum	25	nd	964	1839	1627	nd	nd	nd	nd	nd	191	5539	nd
P. camemberti	25	nd	1732	2026	4441	nd	nd	nd	nd	576	2395	8974	333
P. nalgiovense	25	nd	nd	2393	292	nd	nd	nd	nd	nd	nd	324	412
P. roqueforti	25	nd	450	2351	4478	nd	198	nd	nd	224	2936	3239	603
Blind	25	571	1307	4433	6429	nd	1394	nd	281	947	1946	13622	475

[a]Isolates denoted 1 correspond to experiment 1.
[b]Isolates denoted 2 correspond to experiment 2.
nd, not detected.

Table 5
Number of detected peaks for samples extracted from cultures on CYA

Starter	Temp. (°C)	*P. discolor* 1[a]	*P. discolor* 2[b]	*A. versicolor* 1	*A. versicolor* 2	*P. coprophilum*
G. candidum	18	8	5	0	0	9
P. camemberti	18	10	7	3	5	8
P. nalgiovense	18	6	2	1	3	7
P. roqueforti	18	9	8	1	2	8
Blind	18	10	8	9	13	13
G. candidum	25	7	5	0	2	7
P. camemberti	25	6	9	0	7	8
P. nalgiovense	25	5	2	0	2	3
P. roqueforti	25	7	10	0	6	7
Blind	25	10	10	11	14	10

[a]Isolates denoted 1 correspond to experiment 1.
[b]Isolates denoted 2 correspond to experiment 2.

model with the species *A. versicolor*, *P. discolor* and *P. coprophilum* (Table 5) could be obtained ($R^2 =$ 93%). A PLS model from these data shows that *P. nalgiovense* was the only significant factor describing the variation obtained by both components 1 and 2. The effect of temperature on metabolite production was almost negligible as this factor was located very close to the origin (data not shown).

The HPLC of extracts from Camembert cheese showed 0–3 peaks. Griseofulvin from *P. coprophilum* was the only known metabolite detected. This mycotoxin was detected in all the samples without or with the starters except in the sample from *G. candidum* incubated at 25°C (Table 4). It was not possible to construct any significant models of metabolite production on Camembert cheese. The lower amount and smaller number of mycotoxins detected in cheese compared to a synthetic medium optimal for fungal growth corresponds with the observations of Engel (1978).

The reduction of secondary metabolites by the presence of *P. nalgiovense* on a laboratory medium (CYA) was probably due to the reduction of growth of the mycotoxin producer by this fungus. However, this was not observed on Camembert cheese, for which no correlation was observed between production of griseofulvin by *P. coprophilum* and growth of *P. coprophilum* in the presence of the starters.

In conclusion, the fungal starters showed different competitive abilities on laboratory media and Camembert cheese. Results from inoculation on CYA showed that the presence of *P. nalgiovense* reduced the radial growth as well as the metabolite production of the fungal contaminants. On Camembert cheese *G. candidum* showed a significant effect on growth of the fungal contaminants as growth of all these fungi were totally inhibited at 25°C.

In general, a reduction of secondary metabolites was observed in the presence of the starters that caused growth inhibition of the mycotoxin producer. However, there were no significant changes in metabolite production of the contaminants in the presence or absence of the starters on Camembert cheese.

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