



POTENTIAL PRODUCTION OF CYCLOPIAZONIC ACID BY *PENICILLIUM CAMEMBERTI* STRAINS ISOLATED FROM CAMEMBERT TYPE CHEESE

Miroslava Cíсарová, Dana Tančinová*, Zuzana Barboráková, Zuzana Mašková,
Soňa Felšöciová, Vladimíra Kučerková

Address:

Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department
of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra

*Corresponding author: dana.tancinova@uniag.sk

ABSTRACT

The aim of this study was to isolate the strains of fungi from Camembert type cheese, identify them and to test isolated strains of *Penicillium camemberti* for their ability to produce cyclopiazonic acid. The description of micro- and macromorphological features was used for identification of *Penicillium camemberti* strains. Strains were subsequently *in vitro* tested on their potential ability to produce mycotoxin cyclopiazonic acid (CPA). All of the 14 strains of *Penicillium camemberti*, which were obtained from 20 samples of Camembert type cheese, were cultivated 7, 14, 21, 27 and 30 days on CYA medium at 10±1°C, 15±1°C and 25±1°C in the dark. For determination of CPA production ability by *P. camemberti* isolates *in vitro* was TLC used. After 7 days of cultivation cyclopiazonic acid was produced only by 5 from 14 strains cultivated at all cultivation temperatures. After 14 and 21 days of cultivation was CPA produced by 6 strains at all of cultivation temperatures. After 27 and 30 days of cultivation was CPA identified in 7 strains cultivated at all temperatures of cultivation. The other strains also produced mycotoxin, however, not at each temperature. The most productive at all temperatures and after all days were 5 out of 14 tested strains (S9, S10, S13, S18 and S19). Strains S6 and S16 did not produce CPA at any temperature. The lowest production after all days of cultivation was found at 10±1 °C (44%) and the highest at 25±1 °C (85%).

Keywords: *Penicillium camemberti*, mycotoxin, cheese, cyclopiazonic acid

INTRODUCTION

Penicillium camemberti produces gray-white colonies and is used as a secondary cheeses culture for mold surface-ripened cheeses such as Brie, Camembert and Coulommier. *Penicillium camemberti* is also sometimes referred to as *Penicillium candidum*, *P. caseicolum* or *P. album* (Fuquay et al., 2011). In cheese products, *Penicillium camemberti* and *Geotrichum candidum* are often used because they play a very important role in the formation of flavor (Kinderler, 1989; Jollivet et al., 1994). *Geotrichum candidum* is fungus-like yeast specifically contributes to the cheese with aroma but it can also act as contaminant (Decker et Nielsen, 2005). Filamentous fungi produce a various secondary metabolites – small molecules that are not necessary for normal growth or development (Fox et Howlett, 2008). *Penicillium camemberti* produces cyclopiazonic acid (CPA) (Hozapfel, 1968) on synthetic media (Frisvad et Samson, 2004). Although most studies have reported that the toxin does not occur in cheese, not all agree (Pitt et Hocking, 1997). It has been detected in long and medium ripening cheese (Engel et Teuber, 1989). The presence of CPA in these kinds of cheeses is correlated to the toxigenic ability of starter fungal strain selected (Zambonin et al., 2001). The toxicity of CPA in many animal species has been studied. CPA causes weight loss, diarrhea, degeneration and necrosis of the muscles and viscera, and convulsion and death in rodents (Morrissey et al., 1985; Purchase, 1971), birds (Doner, 1983), dogs (Nuehring et al., 1985) and swine (Lomax et al., 1984). CPA has been implicated in two acute mycotoxicoses in humans: “Kouda poisoning”, for which the kodo millet produced symptoms of giddiness and nausea in man (Rao et Husain, 1985) and “Turkey X disease”, for which CPA was considered to be responsible in addition to aflatoxins (Blount, 1961; Cole, 1986; Sargent et al., 1961).

The objective of our study was to isolate the strains of *Penicillium camemberti* from Camembert type cheese and to test them *in vitro* on their ability to produce cyclopiazonic acid.

MATERIAL AND METHODS

Cheese samples and microorganisms

A total of 20 samples of Camembert type cheese (Table 1) from different European countries, collected from different markets in Slovakia in 2012, were used for *Penicillium camemberti* isolation. A potential toxigenic ability of *Penicillium camemberti* strains obtained from the cheese was tested by thin layer chromatography (TLC).

Isolation and identification of *Penicillium camemberti* strains from cheeses

The microscopic filamentous fungi (*Penicillium camemberti*) from the crust of the peripheral white zone of all cheese samples were inoculated. As cultivation medium MEA (Malt Extract Agar) (Pitt et Hocking, 1997) was used and cultivation at 25±1°C, 5-7 days in darkness was done. After first isolation, *Penicillium camemberti* strains from different cheese samples were inoculated on identification media MEA (Malt Extract Agar) (Pitt et Hocking, 1997), CYA (Czapek Yeast Extract Agar) (Pitt, 1979), YES (Yeast Extract Sucrose Agar) (Samson et al., 2004) and CREA (Creatine Sucrose Agar) (Frisvad, 1985) and cultivated at 25±1°C for 7 days in the dark. The description of micro- and macromorphological features by Samson et al. (2002, 2004) was used for identification of *Penicillium camemberti* strains.

Detection of the potential toxigenicity of *Penicillium camemberti* strains by TLC *in vitro*

All of the 14 strains of *Penicillium camemberti*, which were obtained from 20 samples of Camembert type cheese, were cultivated 7, 14, 21, 27 and 30 days on CYA medium at 10±1°C, 15±1°C and 25±1°C in the dark for testing of their ability to produce cyclopiazonic acid (CPA). CPA was screened by the method adapted from Samson et al. (2002) and modified by Labuda et Tančinová (2006). A three small pieces (each 5x5 mm) were cut from the colony growing on CYA and placed into 1.5 ml Eppendorf vials. Then 500 µl of extraction solvent (chloroform:methanol, 2:1, v/v) was added to the vials containing the agar plugs and shaken on a vortex for at least 2 minutes. The extracts (30–50 µL) were applied afterwards as spots to the TLC plate (Silicagel 60, Merck, Germany) 1 cm apart. Consequently the spots were dried and the plates were developed in a solvent system toluene:ethylacetate:formic acid (6:3:1, v/v/v) that gave an average R_f value of 0.58-0.90 for

CPA. The presence of the CPA was visualized by spraying with Ehrlich reagent (Lund, 1995) and after drying was detected as a violet tailing-spot in daylight.

Table 1 Informations about the investigated samples of Camembert type cheese

Sample	Vendor, town	Commercial name of the cheese	Country of origin
1	Tesco, Dubnica nad Váhom	Président-Karel IV.	Czech republic
2	Tesco, Dubnica nadVáhom	Mäkký zrejúci polotučný syr s bielou plesňou na povrchu	Slovakia
3	Tesco, Dubnica nadVáhom	Král sýrů-Hermelínek	Czech republic
4	Tesco, Dubnica nad Váhom	Camembert	Slovakia
5	Tesco, Dubnica nad Váhom	Plesnivec-tatranský camembert	Slovakia
6	Tesco, Dubnica nad Váhom	Creamfields-Camembert creamy	Poland
7	Tesco, Dubnica nad Váhom	Président-Camembert light 12%	Poland
8	Lidl, Nitra	From´dor Camembert 45%	Germany
9	Lidl, Nitra	From´dor Camembert 13%	Germany
10	Lidl, Nitra	Le Chêne d´argent Camembert	France
11	Coop Jednota, Nitra	Syr plesňový	Slovakia
12	Hypernova, Nitra	Albert quality syr s bílou plísní	Slovakia
13	Hypernova, Nitra	Sedlčanský smetanový Hermelín	Czech republic
14	Billa, Nitra	Clever pleňový syr	Slovakia
15	Billa, Nitra	Révérénd le crèmeux 60%	France
16	Billa, Nitra	Mateda-Kamadet	Czech republic
17	Lidl, Nitra	Brie Chêned´argent 60%	France
18	Lidl, Nitra	Cavabel Weichkäse	Germany
19	Tesco, Nitra	Creamfileds-Brie creamy	Poland
20	Tesco, Nitra	Král sýrů-Modralín	Czech republic

RESULT AND DISCUSSION

Detection of *Penicillium camemberti*

None growth of *Penicillium* was observed in cheese samples no. 1, 4, 7, 12, 14. Only *Geotrichum candidum* was detected in these samples (1, 4, 7, 12, 14). In sample no. 15 were detected *Geotrichum candidum* and *Penicillium caseifulvum*. *Geotrichum candidum* is naturally occurring in raw milk, although generally in low proportions and in cheeses made from the raw milk of various species (cows, sheep and goats) (Boutrou et Guéguen, 2005; Boutrou et al., 2006). It is responsible for the appearance of the cheese, imparting a uniform, white and velvety coat of the surface, but sometimes it can also act as contaminant (Guégen et Schmidt, 1992). It has long been a problem in the canning and freezing industries. Known as “machinery mould” (Eisenberg et Cichowicz, 1977), it is a frequent contaminant of processing lines and consequently of products such as frozen foods (Pitt et Hocking, 1977). Kure et al. (2004) concluded that air was the major source of *Geotrichum candidum* contamination in cheese factories in Norway. *Penicillium camemberti* is closely related to *Penicillium caseifulvum*, which has been isolated from blue cheese, where it acts as spoiler responsible for yellowish spots appearing on the surface of the cheese as well as during the production (Lund et al., 1998; Suhr et al. 2002). *Penicillium camemberti* grows at 5 °C, but not at 37 °C and has pH grown range of 3.5-8.5. In the case of Camembert type cheese, the surface and internal pH values at the end of ripening are about 7.0 and 6.0, respectively (Fuquay et al., 2011). Colonies on CYA (Czapek Yeast Extract Agar) have a diameter of 25-35 mm, occasionally smaller, plane or lightly radially sulcate, convex, floccose. Mycelium is white, pale grey, green or in some isolates persistently white, greyish green and clear exudate sometimes presents. Reverse is pale, yellow or weakly reddish brown (Pitt et Hocking, 2009). On the basis of macro- and micromorphological features (Figure 1) and culture characters we identified all of *Penicillium camemberti* strains.



Figure 1 *Penicillium camemberti* – conidiophore and conidia

Detection of cyclopiazonic acid

Mycotoxins are mold metabolites that are toxic to other living systems and mold growth does not correlate with toxin production. Environmental factors (moisture, oxygen, temperature and type of substrate) appear to determine whether or not mycotoxins are produced (Meerdink, 2002). In this study we monitored effect of temperature and time on cultivation and ability of *Penicillium camemberti* strains to produce cyclopiazonic acid (CPA). All of the 14 strains of *Penicillium camemberti*, which were obtained from 20 samples of Camembert type cheese, were tested for production of cyclopiazonic acid.

For determination of CPA production ability of *Penicillium camemberti* isolates *in vitro* was TLC method used. TLC is a qualitative method for detection of mycotoxins presence in cultivation medium. Results are presented in a Table 2.

At temperature $10\pm 1^{\circ}\text{C}$ after 7 days of cultivation was CPA produced by 36% of tested strains. After 14 and 21 days was CPA production increased on 42%. The highest production of CPA at $10\pm 1^{\circ}\text{C}$ (50%) was observed after 27 and 30 days. At $15\pm 1^{\circ}\text{C}$ was production of CPA after 7 days 42%, but after 14 and 21 days was increased up to 57%. After 27 and 30 days was the highest production (78%). At temperature $25\pm 1^{\circ}\text{C}$ was production after all days of incubation 85%. The most production at all temperatures and after all days was found in 5 out of 14 tested strains (S9, S10, S13, S18 and S19). Only strains S6 and S16 did not produce CPA at any temperature.

Bockelmann et al. (1998) studied sporulation of *Penicillium camemberti* in submerged batch fermentation. Temperature was set to 25 °C at pH 5.6. CPA was produced during fermentation. The observed levels (0.5-4 ppm at 96 h) were strain specific. It can grow at 5°C, but not at 37°C (**Fuquay et al., 2011**).

Taniwaki et al. (2001) in their study with *P. commune* found that CPA was produced in cheese at 25°C after 14 days. This toxin was not synthesized in cheese by *P. commune* at 8-10°C after 1 month (**Still et al., 1979**), suggesting its formation is unlikely under refrigerated storage. **Zambonin et al. (2001)** proved in their study that concentration of CPA in the analysed white surface cheeses ranged from 20 to 80 ppb. The detection limit obtained in cheese (calculated as three-fold the standard deviation of cheese sample at a contamination level of 20 ppb) was 7 ppb. **Hermansen et al. (1983)** studied 62 isolates of *Penicillium* and *Aspergillus* for CPA production by surface and submerged culture on different media. The production of this mycotoxin was restricted to *Penicillium camemberti* group II (and its domesticated form *P. camemberti*), *P. griseofulvum* and *Aspergillus flavus* (and its domesticated form *A. oryzae*). The best yield of CPA was obtained by a strain of *P. griseofulvum*, but several strains of *P. camemberti* group II were also good producers. The best yields of CPA were obtained in submerged culture, but in some cases growth and CPA production occurred only in surface culture. **Lee Bars (1979)** found CPA in 11 out of 20 Camembert cheese crusts in concentration from 0.05 to 1.5 µg.g⁻¹, but this toxin was not found in the inner parts of the cheese. CPA detected in 6 samples of Italian Taleggio, a soft smear-ripened cheese, was confined mainly to the rind also (**Finoly et al., 1999**).

Table 1 The cyclopiazonic acid production by *Penicillium camemberti* strains, cultivated on CYA (Czapek Yeast Extract Agar) *in vitro* in different conditions (temperature and length of cultivation)

Strain	Length and temperature of cultivation														
	7 days			14 days			21 days			27 days			30 days		
	10 °C	15 °C	25 °C	10 °C	15 °C	25 °C	10 °C	15 °C	25 °C	10 °C	15 °C	25 °C	10 °C	15 °C	25 °C
S2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S3	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+
S5	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+
S6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S8	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+
S9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S11	-	-	+	-	-	+	-	-	+	-	+	+	-	+	+
S13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S17	-	-	+	-	-	+	-	-	+	-	+	+	-	+	+
S18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S20	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+

Legend : + mycotoxin confirmed, - mycotoxin unconfirmed, S - strain

CONCLUSION

This study provides a conclusion, that samples of Camembert type cheese were contaminated with other species of fungi besides *Penicillium camemberti*. *P. camemberti* did not grow in samples no. 1, 4, 7, 12, 14 and 15. In these samples were found only strains of *Geotrichum candidum*, which is known as a machinery mould. This mould is a very common problem in raw milk in Europe especially when it is used in the manufacture of soft, fresh cheeses. The obtained strains were tested for the ability to produce cyclopiazonic acid by TLC method. The lowest production after all days of cultivation was found at 10±1 °C (44%) and the highest at 25±1 °C (85%). The most productive strains seemed to be S9, S10, S13, S18 and S19. Strains S6 and S16 did not produce cyclopiazonic acid at any temperature. Mycotoxin levels reported in cheese are not usually considered to be of public health significance. Mouldy cheese is unsuitable for sale and for manufacturing purposes. Protection from the penicillia relies on clean production conditions, low temperature storage, low oxygen atmospheres, integrity of packing materials, intact rinds, preservative impregnated wrappers and rapid turnover of stock.

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