Lait (1998) 78, 661-672

Original article

Penicillium species during the manufacturing and ripening of Armada raw goat's milk cheese: identification, characteristics and in vitro potential toxins production

María E. Tornadijo[a], José M. Fresno[a], Roberto Martín Sarmiento[a], Javier Carballo[b]*

[a] Departamento de Higiene y Tecnología de los Alimentos, Universidad de León, 24071 León, Spain
[b] Area de Tecnología de los Alimentos, Facultad de Ciencias de Orense, Universidad de Vigo, 32004 Orense, Spain

(Received 12 May 1997; accepted 25 May 1998)

Abstract — The species and characteristics of the moulds of the *Penicillium* genus were studied throughout the manufacturing and ripening of Armada cheese. Along the ripening of four batches of cheese, samples of milk, curd, and 1-, 2-, 4-, 8-, and 16-week-old cheese were studied. Of the 274 isolates obtained from the Oxytetracycline Glucose Yeast-Extract Agar medium throughout the manufacturing and ripening, 28 were considered moulds belonging to the *Penicillium* genus and were identified using both morphological and physiological tests as following: *Penicillium roqueforti* (10 strains), *P. crustosum* (6 strains), *P. commune* (4 strains), *P. aurantiogriseum* (3 strains), *P. verrucosum* chemotype I (2 strains), and *P. chrysogenum* (3 strains). The presence of the species of the *Penicillium* genus was more important in the last stages of ripening. This fact could be related to the marked increase in the content of total free fatty acids, and of short-chain fatty acids in particular, which is observed after the second month of ripening in this cheese. The mycotoxin production was investigated by thin-layer chromatography techniques. All the strains were toxigenic in vitro. © Inra/Elsevier, Paris.

Armada goat cheese / mould / *Penicillium* / mycotoxin

Résumé — Étude des espèces du genre *Penicillium* pendant la fabrication et l'affinage du fromage de chèvre au lait cru de la variété Armada. Identification, caractéristiques et capacité de production de toxines in vitro. Nous avons étudié les espèces et les caractéristiques des moisissures du genre *Penicillium* pendant l'élaboration et la maturation de quatre fabrications de fromage Armada.

* Correspondence and reprints.

Dans les quatre fabrications, des échantillons de lait, de caillé et de fromage âgé de 1, 2, 4, 8, et 16 semaines ont été analysés. Des 274 souches isolées à partir du milieu OGYEA, 28 ont été identifiées comme moisissures appartenant au genre *Penicillium*. Elles se répartissent ainsi : *Penicillium roqueforti* (10 souches), *P. crustosum* (6 souches), *P. commune* (4 souches), *P. aurantiogriseum* (3 souches), *P. verrucosum* chimiotype I (2 souches) et *P. chrysogenum* (3 souches). La présence d'espèces du genre *Penicillium* est plus importante à la fin du processus d'affinage, ce qui semble être en rapport avec la forte augmentation de la teneur en acides gras totaux, en particulier à courte chaîne, que l'on observe dans ce fromage à partir du deuxième mois d'affinage. La production de mycotoxines a été mise en évidence par chromatographie sur couche mince. In vitro, toutes les souches se révélaient toxinogènes.

fromage de chèvre Armada / moisissure / *Penicillium* / mycotoxine

1. INTRODUCTION

Moulds are very ubiquitous microorganisms which are found in the majority of food. They have a high metabolic activity and can produce positive and negative effects.

Moulds are used as starter cultures in the elaboration of certain food such as cheese, other dairy products, sausages and cured ham. A lot of cheeses are ripened by moulds, with the species of the genus *Penicillium* (*P. roqueforti*, *P. camemberti*, *P. nalgiovense* and *P. chrysogenum*) being the most used species [20]. The proteolytic and lipolytic enzymes of moulds participe markedly in the ripening of a certain number of cheeses. Proteolysis is needed for the development of an adequate texture and the characteristic flavour. The resulting amino acids are forerunners of aldehydes, alcohols and esters. They can, at the same time, produce desaminations of amino acids; the free ammonia equally contributes to the formation of the aroma. The lipolytic activity of the moulds is also important in the ripening of certain types of cheeses. The free fatty acids generated in the degradation process of the lipids have their own taste. Moulds are also capable of oxidizing fatty acids to metil ketones and of producing from these secondary alcohols.

Moulds are also important as they are responsible for undesirable effects in cheese. They are one of the main causes of spoilage in cheese, provoking musty flavours and an unsightly appearance [1].

Furthermore, some moulds are also able to produce mycotoxins in the cheese dangerous for the consumer. As starter cultures, those strains which do not produce mycotoxins, at least the known ones, are preferred as some of them are highly toxigenic.

This work aims at the study of species and characteristics of moulds of the *Penicillum* genus present in Armada cheese, a variety of Spanish goat's cheese made from raw milk, the role that these moulds plays in the ripening as well as their capability of producing mycotoxins in synthetic culture media since this criterion is taken into account when selecting the strains used as starter cultures.

2. MATERIALS AND METHODS

2.1. Cheese samples

Four batches of Armada cheese were elaborated by experienced cheesemakers following the traditional methods as described by Tornadijo et al. [31]. From each batch, milk, curd, and 1-, 2-, 4-, 8- and 16-week-old cheese samples were taken. Each cheese sample was made up of one whole cheese. Samples were transported to the laboratory under refrigeration (below 5 °C) and analyzed on arrival.

2.2. Microbiological analysis

Fifty g of each sample (after discarding the rind of the cheeses) were homogenized with 200 mL of a sterile solution of 2 % sodium citrate at 40–45 °C for 1 min in a Stomacher 400 Lab Blender (Seward Medical, London, England), thus making a 1/5 dilution. Consecutive decimal dilutions were prepared by mixing 10 mL of the previous dilution with 90 mL of 0.1 % sterile peptone water. One mL of each dilution was inoculated in duplicate in 20 mL of Oxytetracycline Glucose Yeast-Extract Agar (Oxoid) [22] and mixed before solidification. Plates were incubated for 5 days at 22 °C.

2.3. Isolation and identification of strains

From the plates with 30 to 300 colonies, 10 random colonies were taken from each sampling point with aid of a Harrison disc [15]. The 274 isolates obtained were purified and maintained in Sabouraud dextrose agar (Oxoid) tubes under 4 °C covered with sterile parafin. From the 274 isolates obtained, 28 were considered moulds of the *Penicillium* genus. Moulds were firstly diferenciated from yeasts with regard to morphology and type of growth. The moulds were grown in three different media: Czapek agar, Czapek yeast autolysate extract agar (CYA) and Malt extract agar (MEA). In each one of these media the moulds acquired a characteristic morphology. After 7 d of incubation at 25 °C, each of the cultures were macroscopically examinated and the diameter of the colonies, the colour, the texture, the pigment production and the exudate production were noted.

For the microscopic examination, preparations were carried out by taking a portion of the mycelium with a sterile needle and using lactophenol, lactophenol-picric acid and lactophenol-blue cotton as colourants.

2.3.1. Identification at genus level

In order to identify the isolates at genus level, the Samson and van Reenen-Hoekstra [29] methods were followed. The following characteristics were observed in each isolate: the presence of septed mycelia and the production of asexual spores within sporangium or from hypha, the presence of fruit-bodies, the way of conidia formation (from conidiogenous cells or by fragmentations of the hyphae), the disposition of the conidiophore and the form of the conidia.

2.3.2. Identification at species level

The identification at species level of the moulds of the genus *Penicillium* was carried out by examinig the morphological and physiological characteristics. The study of the macro and microscopic morphological characteristics allowed for a previous identification at species level following the methods of Raper and Thom [27], Ramírez [26], Pitt [25] and Samson and van Reenen-Hoekstra [29]. This identification was later confirmed using physiological tests. After incubation at 25 °C for 7 d in the Czapek agar, CYA and MEA media, macroscopic characteristics were observed (colour; texture (velvetlike, fasciculate, woollike, funiculose); smell; exudate production; pigment production; diameter of the colonies) and microscopic characteristics (structure of the conidiophore (grade of ramification between phialide and stipe); form and dimensions of the phialide, metula and rami; surface of the stipe (smooth, rough, verrucose); and size, surface and colour of the conidia.

In the genus *Penicillium* some isolates are usually difficult to identify only with regard to morphological characteristics as some criteria such as the texture and the colour are fairly subjective. Some especies are also difficult to identify observing their microscopic characteristics. For a better identification, in agreement with the criteria of Frisvad [10] and Pitt [25], the following physiological characteristics were studied: reaction with $FeCl_3$; ability to produce rottenness in apples and/or in citric fruits; milk digestion; growth in creatine-sucrose agar (CREA); growth in agar and in solution with NO_2^-; growth at 5, 30, 34 and 37 °C; inhibition of growth of *Staphylococcus aureus*, *Candida spp.*, *Bacillus cereus* and *Bacillus subtilis*; lipolytic activity (growth on tricaproin and tributyrin agar); growth in presence of fungicides using the following culture media: GYBS (Glucose Yeast extract agar with 50 ppm of sorbic acid and 50 ppm of benzoic acid), GYP (GY agar with 1 000 ppm of propionic acid), and GYA (GY agar with 5 000 ppm of acetic acid).

2.4. Production of mycotoxins by the *Penicillium* species

The strains of the genus *Penicillium* were tested for the production of mycotoxins since those strains which do not produce any known toxins are prefered as starter cultures. Moreover, accord-

ing to Frisvad and Filtenborg [11] the terverticillate Penicillia can be defined according to their secondary metabolite profiles as they can be used as a taxonomic criterium. The strains were grown in different media (CYA, OA and YES) at 25 °C for 7–14 d. In order to produce the mycotoxins, two techniques were followed: the one of toxins extraction [10] and the one of the agar top [6, 7]. The identification of the mycotoxins was carried out by thin-layer chromatography techniques using TLC aluminium plates silica gel 60 WF_{264}s from Merck and specific revelations. Griseofulvin, mycophenolic acid, penicillic acid, roquefortine and penitrem A were used as standards at 0.5 $mg \cdot mL^{-1}$. Some tests were carried out by previously impregnating the plates in oxalic acid at 8 % in water or in methanol, air-drying them afterwards; better results were obtained with the acid mycotoxins using this method.

The mixtures TEF (toluene/ethylacetate/90 % formic acid (5:4:1)) and CAP (chloroform/acetone/propane-2-ol [85:15:20]) were used as eluants. Once the runs had finished, the plates (before and after the treatment with different reagents) were observed under visible and ultraviolet at 254 nm lights; some mycotoxins were visible without any type of treatment. The plates were developped with chemical reagents pulverization or exposition to their vapours. Mycotoxins appeared as coloured or fluorescent spots [7, 10, 11, 29]. The used reagents with the plates eluted with TEF and the mycotoxins which were visualized with each one of them were the following:

– reagent 1 (R_1): 1 % $FeCl_3$ (w/v) in butane-1-ol. It allowed us to visualize griseofulvin, mycophenolic acid, PR-toxin, roquefortine C, and ochratoxin A;

– reagent 2 (R_2): 50 % H_2SO_4 in water, heating for 5 min at 120–130 °C; it allowed us to visualize griseofulvin, penicillic acid, mycophenolic acid, PR-toxin, penitrem A, rugulovasine, cyclopiazonic acid, and cyclopenol/cyclopenin;

– reagent 3 (R_3): ANIS (0.5% p-anisaldehyde in methanol or in ethanol/acetic acid/concentrated sulphuric acid [17:2:1]), heating the plate for 5 min at 120 °C; it allowed us to visualize above all griseofulvin, mycophenolic acid, penicillic acid, penitrem A, roquefortine C, and terrestric acid;

– reagent 4 (R_4): exposure to NH_3 vapours for 1–3 min; after this, plates were pulverized with ANIS and heated; it allowed us to visualize griseofulvine and penicillic acid;

– reagent 5 (R_5): 20 % $AlCl_3$ (w/v) in 96 % ethanol, the plates were then heated at 120 °C for 5 min. It allowed us to visualize penitrem A;

– reagent 6 (R_6): 1 % $FeCl_3$ (w/v) in buthane-1-ol and ANIS heating the plates at 120 °C for 10 min. It allowed us to visualize griseofulvin, penitrem A, roquefortine C, rugulovasine, and cyclopiazonic acid.

For the plates eluted with CAP the reagent 7 (R_7) was used: 1 % $Ce(SO_4)_2$ in H_2SO_4 6N. It allowed us to visualize roquefortine C, griseofulvin, penitrem A, mycophenolic acid, penicillic acid, rugulovasine, and cyclopenol/cyclopenin.

To identify the mycotoxins the absolute Rf and the relative Rf to griseofulvin (Rfg) were measured.

3. RESULTS

Of the 274 strains obtained from the OGYEA medium during the manufacturing and ripening of the four batches of Armada cheese, twenty eight were appointed to the genus *Penicillium*. Of the characteristics which are shown, a green or white mycellium with a good sporulation, conidiophores in an artist's brush form (*penicillium*), presence of conidiogenous cells (phialides), metulae and rami stand out. The phialides are bottle-shaped or, if longer, spear-shaped. The conidia come from these through basipetal sucession.

The identification at the species level of the 28 strains of the genus *Penicillium* was as following.

Ten strains were identified as *P. roqueforti*; the identification of these strains did not pose any great difficulty since the morphology both macro and microscopically is very characteristic. Three strains were identified as *P. chrysogenum*, and six as *P. crustosum*. Nine strains were included in the group *P. verrucosum* complex according to Samson and van Reenen-Hoekstra [29]; within this group paying attention above all to the physisological characteristics 2 strains were identified as *P. verrucosum* chemotype I, 4 as *P. commune*, and 3 as *P. aurantiogriseum*.

Table I shows the distribution of these species at the different sampling points and their morphological characteristics. Their physiological and cultural characteristics are shown in *table II*.

Table III shows the mycotoxins produced in vitro by the strains of the *Penicillium* genus isolated, their Rfg values using TEF and CAP as eluants and the reagents used in the developing. The 10 *P. roqueforti* strains produced roquefortine C, mycophenolic acid, penicillic acid and PR-toxin. The 3 *P. chrysogenum* strains produced roquefortine C and PR-toxin. Of the 6 *P. crustosum* strains, 1 produced penitrem A, terrestric acid, roquefortine C, and rugulovasine; 3 produced penitrem A, terrestric acid, and roquefortine C; 1 produced roquefortine C, penitrem A, and rugulovasine; the remaining strain produced penitrem A and terrestric acid. The 2 *P. verrucosum* strains produced ochratoxin A, this mycotoxin is exclusively produced by *P. verrucosum* and thus makes up a differential character of this species. Of the 4 *P. commune* strains, 1 produced rugulovasine, penitrem A, cyclopenol/cyclopenin, and cyclopiazonic acid; 1 produced rugulovasine, penitrem A, and cyclopenol/cyclopenin; 1 produced rugulovasine and penitrem A; the remaining strain produced cyclopiazonic acid and cyclopenol/cyclopenin. Lastly, of the 3 *P. aurantiogriseum* strains, 1 produced terrestric acid, rugulovasine and penitrem A; 1 produced terrestric acid, rugulovasine and cyclopiazonic acid; the remaining strain produced terrestric acid and penitrem A.

4. DISCUSSION

The criteria used for the identification of the isolated moulds based both on the morphological and physiological characteristics can be justified because although the taxonomy of the moulds traditionally has been carried out with almost exclusive regard to morphological criteria, both macroscopic and microscopic, within the *Penicillia terverticillata* there exists a great variability which is seen both in macromorphology and in micromorphology; successive cultures of the same strain in the same medium can give rise to colonies of different dimensions and aspects.

The texture of the colonies was one of the criterion used by Raper and Thom [27] to establish taxons in the genus *Penicillium*. This criterion as well as being artificial is very subjective as Ciegler et al. [2] have already established which is why it is very difficult to situate a species into one or another section. Even Raper and Thom [27] considered intergraduations exist between sections, in such a way that a same species could have variations in some characteristics; successive cultures of the same strain could also affect the morphological characteristics of the colonies. Other authors such as Ramírez [26] and Pitt [25] used similar criteria, although Pitt [25] placed more emphasis on the micromorphological characters. Either way important micromorphological differences can not always be observed, as certain asymetric *Penicillia* may have one or more sized branches which end in verticiles of metula and phialides, and conidiophores more or less long which can be smooth, rough or slightly rough [2]. Therefore, complementary tests are needed at least to confirm identification, above all within the group that Samson and van Reenen-Hoekstra [29] described as *P. verrucosum* complex. Many species included in this group were the object of frequent taxonomic confusions [11]. Various authors have begun to establish physiological criteria which could be applied to the taxonomy of moulds. Samson and van Reenen-Hoekstra [29] briefly included this posibility, however there is a lack of available information in order to establish a relationship between the physiological characters and taxons.

With regard to the *Penicillium* species found throughout the making and ripening of Armada cheese predominated *P. roqueforti*

Table I. Morphological characteristics* and distribution in the sampling points of *Penicillium* species isolated during the manufacturing and ripening of Armada cheese (four batches).

Tableau I. Caractères morphologiques* et distribution aux stades d'échantillonnage des souches de moisissures du genre *Penicillium* isolées durant la fabrication et l'affinage du fromage Armada (quatre fabrications).

	P. roqueforti	*P. chrysogenum*	*P. crustosum*	*P. verrucosum* chemotype I	*P. commune*	*P. aurantiogriseum*
No. of isolates	10	3	6	2	4	3
Distribution in the sampling points	milk (2) 16-w-old cheese (8)	curd (1) 2-w-old cheese (1) 8-w-old cheese (1)	8-w-old cheese (1) 16-w-old cheese (5)	curd (1) 8-w-old cheese (1)	4-w-old cheese (1) 16-w-old cheese (3)	2-w-old cheese (1) 16-w-old cheese (2)
Smell	Not detectable	Slight (aromatic)	Noticeable (fruity)	Strong (glue or acetone)	Strong (mouldy) in old cultures	Noticeable (fruity)
Exudate	No	Abundant (yellowish)	Abundant (colourless drops)	Slight (bright drops)	Slight	Slight (colourless drops)
Colour in MEA	Green-grey	Green-grey	Green-grey	Green (white edges)	Green	Green (white edges)
Diameter in MEA	7 cm	2.4–2.5 cm	2.5 cm	1.1–1.2 cm	—	1 cm
Texture in MEA	Velvet-like	Velvet-like	Very sporulated	Cotton-like	—	Velvet-like
Aspect in MEA	Spider's web	Flat	They easily detach masses of conidia	Central area with a dark green colour	—	Not flat
Colour in Czapek	Green-greyish	Green-grey	Greyish-green	White	White (green centre)	White
Diameter in Czapek	1.5–2 cm	—	2.5 cm	1.1 cm	1.5 cm	1 cm
Texture in Czapek	—	Velvet-like	—	Cotton-like	—	Cotton-like
Colour in CYA	Green-grey (white edge)	Green or yellowish green (white edges)	Green (white edge)	White	Green-blue	Green (white edge)
Diameter in CYA	—	3.5–3.7 cm	3 cm	1.5 cm	2–3.5 cm	1.5 cm
Aspect in CYA	Flat with a higher central area	Radially grooved	Radially grooved	Radially grooved	Radially grooved	Radially grooved
Reverse side	Dark green (almost black)	Yellow green or pale yellow	Yellow-cream	Brown-cinnamon halos	Creamy white	Bright yellow-orange

Table I. (Continued). Tableau I. (Suite).

	P. roqueforti	*P. chrysogenum*	*P. crustosum*	*P. verrucosum* chemotype I	*P. commune*	*P. aurantiogriseum*
Conidiophores	Terverticillate	Bi and terverticillate	Bi and terverticillate	Ter and quater-verticillate	Bi and terverticillate	Bi and verticillate
Stipes	Warty	Smooth or slightly rough	Smooth or slightly rough	Finely spinulose	Smooth or finely rough	Smooth or very
Conidia	Fairly large	Elliptic became more globose	Elliptical, globose and subglobose	Globose or subglobose	Globose or subglobose	—
Diameter of conidia	Until 6 µm	3–4 µm	4 µm	2–3 µm	3–5 µm	—

* After incubation 7 d at 25 °C.
* Après incubation de 7 j à 25 °C.

which was isolated from milk and from sixteen-week-old cheese. Obviously, the levels of *P. roqueforti* found in Armada cheese are much lower than those observed in blue-veined cheeses [3, 5, 12, 24]. The presence of this species in the last stages of the ripening of Armada cheese may be explained by the presence of craks in some of the cheeses which ease its implantation in the inside of the mass.

P. roqueforti had an intense proteolytic activity [13], which does not fully agree with the low levels of proteolysis observed in Armada cheese [9]. Possibly the high values of salt/moisture ratios in Armada cheese [8] are responsible to the low activity of the *P. roqueforti* enzymes in this cheese; it is known that the activity of *P. roqueforti* proteases falls as the concentration salt/moisture rises to above 0.5 [18]. On the other hand, a great variability in the proteolytic activity of the *P. roqueforti* strains has been observed, showing that, in general, the strains with a high proteolytic capacity have a low lipolytic capacity and reciprocally [23, 28]. Given the intense lipolysis undergone by Armada cheese [9] and the possible participation of *P. roqueforti* in this process in the last stages of ripening, it is possible that the *P. roqueforti* strains in this cheese have a weak proteolytic activity. The proteolytic capacity of the *Penicillium* species isolated from Armada cheese also could be inhibited by high concentrations of free fatty acids generated during ripening [16].

With regard to the possible participation of *P. roqueforti*, and that of the other species of the *Penicillium* genus isolated, in the lipolytic processes that take place during the ripening of Armada cheese, the presence of these species could be related to the marked increase in the content of total free fatty acids, and of short-chain fatty acids in particular, which is observed after the second month of ripening [9]. The *P. roqueforti* lipases have a very intense activity, characterized by a high production of caprylic acid and other short-chain fatty acids [14]. More-

Table II. Physiological characteristics of the *Penicillium* strains isolated throughout the manufacturing and ripening of Armada cheese.
Tableau II. Caractères physiologiques des souches du genre *Penicillium* isolées au cours de la fabrication et de l'affinage du fromage Armada.

	P. roqueforti	*P. chrysogenum*	*P. crustosum*	*P. verrucosum* chemotype I	*P. commune*	*P. aurantiogriseum*
No. of strains	10	3	6	2	4	3
Rottenness in apples	0	0	6	0	2	0
Rottenness in citric fruits	10	2	0	1	1	1
Milk coagulation	0	3	0	0	0	0
Growth in:						
Tricaproin agar	0	0	0	0	0	0
Tributyrin agar	10	3	6	2	4	3
Growth at 5 °C	0	0	0	0	0	0
Growth at 30 °C	N.D.	N.D.	0	N.D.	0	0
Growth at 34 °C	N.D.	N.D.	0	N.D.	0	0
Growth at 37 °C	0	0	0	0	0	0
Reaction with $FeCl_3$	10 yellow	3 orange	3 yellow, 3 orange	1 yellow, 1 orange	2 yellow	0
Inhibition of:						
Candida spp.	0	0	0	0	0	3
S. aureus	0	3	4	1	0	2
B. cereus	0	3	5	0	3	2
B. subtilis	10	3	5	1	3	2
Growth in CREA	10	3	6	0	4	0
Acid in CREA	0	3	6	0	4	3
Alkali in CREA	0	0	6	0	4	0
Growth in agar NO_2^-	10	0	6	2	0	0
Caseinolytic activity	10	3	6	2	3	3
Growth in:						
GYBS	10	3	6	0	4	0
GYP	10	0	6	0	2	0
GYA	10	0	0	0	0	0

N.D : not determined.
N.D. : non déterminé.

Table III. Relative Rf values to griseofulvin (Rfg)*, visualization and coulours of the mycotoxins produced in vitro by the *Penicillium* strains isolated throughout the manufacturing and ripening of the Armada cheese.

Tableau III. Rf relatif par rapport à la griseofulvine, visualisation et couleurs des mycotoxines produites in vitro par les souches du genre *Penicillium* isolées durant la fabrication et l'affinage du fromage Armada.

Species	Rfg in TEF	Mycotoxins	Developing	Rfg in CAP	Developing	No. of producing strains
P. roqueforti	0.07 – 0.08	Roquefortine C	none R6 – visible	0.22 - 0.21	R7 – red	10/10
	1.26 – 1.34	Mycophenolic acid	none R2 – yellow	1.04 – 1.03	none	10/10
	1.05 – 1.08	Penicillic acid	R2 – yellow	0.83 – 0.88	R7 – blue	10/10
	1.41	PR – toxin	R1			10/10
P. chrysogenum	0.11 – 0.14	Roquefortine C	R6 – orange R1 – brown	0.43 – 0.47	R7 – blue – green	3/3
	1.39 – 1.43	PR – toxin	none R2 – dark	1.11 – 1.13	none	3/3
P. crustosum	0.57 – 0.64 0.72 – 0.80	Terrestric acid	none R3 – yellow			3/6 5/6
	0.05 – 0.06	Rugulovasine	R6 – dark			2/6
	0.07 – 0.14	Roquefortine C	R3 – violet	0.41 – 0.42	none	5/6
	1.51 – 1.55 1.56 – 1.74	Penitrem A	R2 – orange R3 – violet	1.12 – 1.22	R7 – dark	6/6
P. verrucosum chemotype I	1.35 – 1.39	Ochratoxin A	none R1 – blue	0.31	none	2/2

Table III. (continued). Relative Rf values to griseofulvin (Rfg)*, visualization and coulours of the mycotoxins produced in vitro by the *Penicillium* strains isolated throughout the manufacturing and ripening of the Armada cheese.

Tableau III. (suite). Rf relatif par rapport à la griseofulvine, visualisation et couleurs des mycotoxines produites in vitro par les souches du genre *Penicillium* isolées durant la fabrication et l'affinage du fromage Armada.

Species	Rfg in TEF	Mycotoxins	Developing	Rfg in CAP	Developing	No. of producing strains
P. commune	0.05 – 0.08	Rugulovasine	R6 – dark R2 – blue	0.08	R7 – blue 2/3	1/3
	1.51 – 1.60 1.55 – 1.70	Penitrem A	R2 – orange R3 – violet	1.09 – 1.14	none	3/3
	1.30 – 1.39	Cyclopiazonic acid	none R2 – blue R6 – blue			2/3
	1.03 – 1.05	Cyclopenol/Cyclopenin	none Oxalic – R2 – blue	0.84 – 0.92	R7 – blue	3/3
P. aurantiogriseum	1.51– 1.55	Penitrem A	R2 – orange R3 – violet	1.09– 1.13	none	2/3
	0.58 – 0.69	Terrestric acid	none			3/3
	1.39	Cyclopiazonic acid	none			1/3
	0.05 – 0.06	Rugulovasine	R6– dark			2/3

* Rf values of griseofulvin were 0.36–0.38 in TEF and 0.65–0.70 in CAP. Eluants: TEF: toluene/ethylacetate/90% formic acid (5:4:1). CAP: chloroform/acetone/propane-2-ol (85:15:20).

* Les valeurs du Rf de la griseofulvine furent 0,36–0,38 avec TEF et 0,65–0,70 avec CAP.

over, this activity is not seen to be strongly inhibited by low pH values above 5 [30] or by high salt/moisture concentrations above 8% [17].

All the isolated *Penicillium* strains produced in vitro any mycotoxin. Our results corroborate the findings of other authors; according to information reported by Leistner [20], 75% of the *Penicillium* strains isolated from foods and the majority of the *Penicillium roqueforti* strains produce some mycotoxin.

However, the capacity to produce mycotoxins is very different in cheese and in synthetic culture media. Eventhough moulds find a good medium of growth in cheese, it does not seem adequate for the production of mycotoxins due its chemical composition [4]. Some mycotoxins are produced in low quantities or they either they become unstable in cheese, thus reducing their biological activity. Some authors associate this fact with the high protein content in cheese [21], the toxins can combine with amino acids and other compounds (-SH radicals, ammonia) and thus become inactive [19]. The production of mycotoxins can also be influenced by the Aw and the temperature of the medium [19]. The Aw values of cheese are not adequate for the production of these metabolites, nor its temperature since cheeses before being sold are usually kept in refrigeration.

ACKNOWLEDGEMENTS

This study was financially supported by the Junta de Castilla y León (the Regional Government). Author M E Tornadijo was awarded a grant from the Consejería de Agricultura y Ganadería of the Junta de Castilla y León.

REFERENCES

[1] Choisy C., Guéguen M., Lenoir J., Schmidt J.L., Tourneur C., L'affinage du fromage. Chap. 1: Les phénomènes microbiens, in: Eck A. (Coord.), Le Fromage, Tech & Doc Lavoisier, Paris, 1987, pp. 190–259.

[2] Ciegler A., Fennell D.I., Sansing G.A., Detroy R.W., Bennett G.A., Mycotoxin-producing strains of *P. viridicatum*, classification into subgroups, Appl. Microbiol. 26 (1973) 271–278.

[3] De Boer E., Kuik D., A survey of the microbiological quality of blue veined cheeses, Neth. Milk Dairy J. 41 (1987) 227–237.

[4] Dragacci S., Mycotoxines, in: Eck A., Gillis J.C. (Coord.), Le Fromage, 3rd. ed., Lavoisier Tec & Doc, Paris, 1997, pp. 781–784.

[5] El Dairouty R.K., Abd Alla E.S.A.M., El Senaity M.M., Tawfek N.F., Sharaf O.M., Chemical and microbiological changes in Roquefort style cheese during ripening, Ecol. Food Nutr. 24 (1990) 89–95.

[6] Filtenborg O., Frisvad J.C., A simple screening-method for toxigenic moulds in pure cultures, Lebensm. Wiss. Technol. 13 (1980) 128–130.

[7] Filtenborg O., Frisvad J.C., Svendsen J.A., Simple screening method for molds producing intracelular mycotoxins in pure cultures, Appl. Environ. Microbiol. 45 (1983) 581–585.

[8] Fresno J.M., Tornadijo M.E., Carballo J., González Prieto J., Bernardo A., Characterization and biochemical changes during the ripening of a Spanish craft goat's milk cheese (Armada variety), Food Chem. 55 (1996) 225–230.

[9] Fresno J.M., Tornadijo M.E., Carballo J., Bernardo A., González Prieto J., Proteolytic and lipolytic changes during the ripening of a Spanish craft goat's milk cheese (Armada variety), J. Sci. Food Agric. 75 (1997) 148–154.

[10] Frisvad J.C., Physiological criteria and mycotoxin production as aids in identification of common asymetric Penicillia, Appl. Environ. Microbiol. 41 (1981) 568–579.

[11] Frisvad J.C., Filtenborg O., Classification of terverticillate Penicillia based on profiles of mycotoxins and other secondary metabolites, Appl. Environ. Microbiol. 46 (1983) 1301–1310.

[12] González de Llano D., Ramos M., Rodríguez A., Montilla A., Juárez M., Microbiological and physicochemical characteristics of Gamonedo blue cheese during ripening, Int. Dairy J. 2 (1992) 121–135.

[13] Gripon J.C., Desmazeaud M.J., Le Bars D., Bergère J.L., Role of proteolytic enzymes of *Streptococcus lactis*, *Penicillium roqueforti* and *P. caseicolum* during cheese ripening, J. Dairy Sci. 60 (1977) 1532–1538.

[14] Ha J.K., Lindsay R.C., Release of volatile branched-chain and other fatty acids from ruminant milk fats by various lipases, J. Dairy Sci. 76 (1993) 677–690.

[15] Harrigan W.F., McCance M.E., Statistical methods for the selection and examination of microbial colonies, in: Harrigan W.F., McCance M.E. (Eds.), Laboratory methods in foods and dairy microbiology, Academic Press, London, UK, 1976, pp. 47–49.

[16] Imamura T., Studies on the changes of milk proteins by *Penicillium roqueforti*, J. Agric. Chem. Soc. 34 (1960) 892.

[17] King R.D., Clegg G.H., The metabolism of fatty acids, methyl ketones and secondary alcohols by *Penicillium roqueforti* in blue cheese slurries, J. Sci. Food Agric. 30 (1979) 197–202.

[18] Kinsella J.E., Hwang D.H., Enzymes of *Penicillium roqueforti* involved in the biosynthesis of cheese flavor, Crit. Rev. Food Sci. Nutr. 8 (1976) 191–228.

[19] Le Bars J., Les moisissures productrices de toxines, in: Hermier J., Lenoir J., Weber F. (Eds.), Les groupes microbiens d'intérêt laitier, CEPIL, Paris, 1992, pp. 509–531.

[20] Leistner L., Mould-ripened foods. Fleischwirtschaft 66 (1986) 1385–1388.

[21] Lieu F.Y., Bullerman L.B., Production and stability of aflatoxins, penicillic acid and patulin in several sustrates, J. Food Sci. 42 (1977) 1222–1224, 1228.

[22] Mossel D.A.A., Kleynen-Semmeling A.M.C., Vincentie H.M., Beerens H., Catsaras M., Oxytetracycline-glucose-yeast extract agar for selective enumeration of moulds and yeasts in foods and clinical material, J. Appl. Bacteriol. 33 (1970) 454–457.

[23] Niki T., Yoshioka Y., Ahiko K., Proteolytic and lipolytic activities of *Penicillium roqueforti* isolated from blue cheese, Proc. 17th. Int. Dairy Congr., Munchen, Germany, Vol D, (1966) 531.

[24] Núñez M., Medina M., Gaya P., Dias-Amado C., Les levures et les moisissures dans le fromage bleu de Cabrales, Lait 61 (1981) 62–79.

[25] Pitt J.I., A laboratory guide to common *Penicillium* species, 2nd. ed., CSIRO, Division of Food Research, North Ryde, NSW, Australia, 1988.

[26] Ramírez C., Manual and atlas of the Penicillia, Elsevier Medical Press, Amsterdam, 1982.

[27] Raper K.B., Thom C., A manual of the Penicillia. Hafner Publishing Company, New York, 1968.

[28] Salvadori P., Bianchi B., Cavalli V., Study on the action of amino acids on Penicillia used in making blue-veined cheese, Lait 44 (1964) 129.

[29] Samson R.A., van Reenen-Hoekstra E.S., Introduction to food-borne fungi, 3rd. ed., Samson R.A, van Reenen-Hoekstra, E.S. (Eds.), Centraalbureau voor Schimmelcultures, Baarn, Netherlands, 1988.

[30] Tomasini A., Bustillo G., Lebeault J.M., Fat lipolyzed with a commercial lipase for the production of blue cheese flavour, Int. Dairy J. 3 (1993) 117–127.

[31] Tornadijo M.E., Fresno J.M., Bernardo A., Martín Sarmiento R., Carballo J., Microbiological changes throughout the manufacturing and ripening of a Spanish goat's raw milk cheese (Armada variety), Lait 75 (1995) 551–570.