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Mycoflora of a traditional Spanish blue cheese

Teresa-María López-Díaz*; Jesús-Angel Santos; Miguel Prieto; María-Luísa García-López; Andrés Otero

Department of Food Hygiene and Food Technology, University of León, 24071 León, Spain

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Summary

The mycoflora of a Spanish blue cheese made in a natural way (Valdeón cheese) was investigated. Moulds and yeasts isolated in cheeses at different stages of manufacture and at the stage of consumption were identified to species level and the proteolytic and lipolytic activities of the major strains estimated. *Penicillium* was the main genus identified, with *P. roqueforti*, *P. verrucosum*, *P. chrysogenum*, the morphologically closely related species *P. aurantiogriseum*/*P. solitum*/*P. commune*, and *P. expansum* being the major species. Other genera found were *Cladosporium*, *Mucor* and to a lesser extent *Paecilomyces*, *Acremonium*, *Alternaria* and *Geotrichum*. The major species of yeasts found were *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Yarrowia lipolytica*. On the surface of the finished cheese *P. aurantiogriseum*/*P. solitum*/*P. commune* and *D. hansenii* were dominant. The most lipolytic species were *P. roqueforti*, *P. verrucosum* and *Y. lipolytica*. *P. chrysogenum* was the most proteolytic. None of the *P. roqueforti* strains showed proteolytic activity.

Keywords: moulds, yeasts, traditional blue cheese.

1 Introduction

Valdeón cheese is a Spanish blue cheese made in a completely natural way. Raw milk is used, no starters are added and the ripening takes place in mountain caves with no control of the environment. Under such conditions, apart from the natural flora of the milk, a high degree of contamination by microorganisms, including fungi, is expected.

Previous studies have shown high levels of moulds and yeasts during the ripening of this hand-made variety (1). The study of this flora is of scientific, technological and even sanitary interest. Fungi may be responsible for undesired effects in cheese. They are among the major causes of spoilage in cheese, mainly musty flavours and unsightly appearance (2). In addition, the ability of a great number of moulds to produce mycotoxins is well known (3). On the other hand, some fungi are useful in cheese technology, a clear example being the manufacture of blue cheeses, where the mould *Penicillium roqueforti* is responsible for the special features of the product (flavour and blue-veined appear-

ance) (4). Traditionally, *Penicillium* inoculation, its growth and that of the secondary flora were natural processes in these varieties. However, when cheese is manufactured on a large scale, it is essential to use carefully selected strains able to yield a good quality cheese.

The aim of this study, part of a wider project, was to investigate the mycoflora of Valdeón cheese as the first step in the selection of strains of moulds and yeasts to be used in an industrial manufacture.

2 Materials and methods

2.1 Samples

Handmade cheeses were obtained from the producer villages (Cain and Cordiñanes, Valdeón valley, León, Spain). The cheesemaking process was briefly as follows. Raw goat's milk (occasionally mixed with cow's milk) was coagulated (20-30 °C for 1-2 h) with animal rennet (kids) or commercial rennet. The whey was removed with a saucepan and the curd was placed into suitable cylindrical moulds (tinplates). Drainage proceeded for two days and during this time coarse salt was sprinkled over the surfaces. During the first 10 to 15 days of ripening (drying) the cheeses were placed in a warm room (17-23 °C and 70-90% relative humidity, RH). For the next stage of ripening, the cheeses were moved to natural mountain caves (700-1500 m altitude; 7-12 °C and 95-99% RH) and turned at intervals throughout the remainder of the ripening period (2-4 months) (5).

Twenty samples of cheese at the stage of consumption were taken for the study of the mycoflora. In addition, the complete manufacturing process of five of these samples was followed and samples were taken at six stages (milk, curd, salted cheese, dry cheese, cheese in the middle of the ripening and finished cheese) to assess the evolution of the mycoflora.

Sampling was performed according to I.C.M.S.F. (6). Curd and cheese samples (25 g from the interior and 10 g from the surface, in this last case only from the drying onwards) were aseptically homogenized in 2% sodium citrate kept at 45 °C. Milk samples (10 ml) were diluted with 0.1% peptone water. Serial 10-fold dilutions were prepared with all the homogenates in 0.1% peptone water and 0.1 ml aliquots of appropiate dilutions were plated onto OGYEA (Oxytetracycline Glucose Yeast Extract Agar, Oxoid) plates. Duplicate plates were incubated at 25 °C for 5 days.

2.2 Mycological analysis

A total of 90 strains of moulds and 68 of yeasts were isolated. After a macroscopic and microscopic examination, representative colonies (at least two) of each type of mould and yeast present in all the plates of each sample were selected and the relative occurrence of each kind of colonies isolated was estimat-

ed. Mould cultures were also directly isolated from the blue veins of ten of the finished cheese samples in the following way. Fragments of mycelium and spores were taken with a sterile loop, using a dissecting microscope, and placed in 1 ml volumes of sterile 0.1% Tween 80. Then, 0.1 ml aliquots of the suspension were plated onto OGYEA plates. The incubation conditions and isolation technique were as indicated before. All the isolates were stored on malt extract agar slopes at 4 °C until identification within six months.

Mould isolates were identified to genus and species level according to Pitt & Hocking (7) and Samson & Reenen-Hoekstra (8). The *Penicillium* strains were identified to species level following the key of Pitt (9), with the modifications of Williams & Pitt (10); the key and description of species by Ramírez (11) and Samson & Reenen-Hoekstra (8) were also used. The classification was subsequently reviewed using the key of Pitt & Cruickshank (12).

Yeast isolates were identified using the Yeast Identification System ATB32C (API System, Vercieu, France). In addition, several confirmatory tests such as lactose fermentation, nitrate assimilation and morphological characteristics (type of asexual reproduction, formation of clamydospores and ballistospores, sexual reproduction and formation of mycelium-pseudomycelium) were performed according to Van der Walt & Yarrow (13) and Barnett et al. (14). The identification was completed using the description of species by Kreger-van Rij (15) and Barnett et al. (14).

2.3 Proteolytic and lipolytic acitivities

Hydrolysis of tributyrin was assayed as described by Harrigan & McCance (16). Proteolytic activity on a medium containing casein was assayed according to El-Gendy & Marth (17). In both cases the activity was determined as the width of the clear zone around the colonies.

3 Results and discussion

The genera and species of moulds found in Valdeón cheese are summarized in Table 1. Nearly 75% belonged to the genus *Penicillium*. Other genera found were *Cladosporium*, *Mucor* and, to a lesser extent, *Paecilomyces*, *Acremonium*, *Alternaria* and *Geotrichum*.

The taxonomy of *Penicillium*, considered as one of the most diverse genera of fungi in nature, is very complex. In the last few years, the use of new criteria such as secondary metabolites and isoenzymes has clarified the taxonomy of this genus, particularly the subgenus *Penicillium*, and has confirmed the validity of morphological and gross physiological properties as taxonomic features (12).

Table 2 shows the classification of the species of *Penicillium* isolated from our samples. Most strains (64/65) were classified in subgenus *Penicillium*. This agrees with the findings of other authors who have observed the dominance of this group in foods (18).

Table 1. Genera and species of moulds found in Valdeón cheese.

Genus	Species	Number of strains	
Penicillium		65	
	P. roqueforti		18
	P. aurantiogriseum/P. commune/P. solitum		14
	P. verrucosum		12
	P. chrysogenum		12
	P. expansum		7
	P. glabrum		1
	P. viridicatum		1
Cladosporium		9	
	C. herbarum		5
	C. cladosporioides		4
Mucor		6	
	M. racemosus		6
Paecilomyces		3	
	P. variotii		3
Acremonium sp.		3	
Alternaria sp.		2	
Geotrichum		2	
	G. candidum		2

Table 2. Taxonomy of *Penicillium* species found in Valdeón cheese, identified according to Williams & Pitt (10), and their reclassification according to Pitt & Cruickshank (12).

	Number of strains	
SUBGENUS *Aspergilloides*	1	
SECTION *Aspergilloides*		
SERIE *Glabra*		
P. glabrum		1
SUBGENUS *Penicillium*	64	
SECTION *Penicillium*		
SERIE *Expansa*	23	
P. chrysogenum		11
P. expansum		7
P. aurantiogriseum		4
P. griseoroseum (*P. chrysogenum*)		1
SERIE *Urticicola*	20	
P. verrucosum		12
P. aurantiogriseum (*P. solitum*)		7
P. olivicolor (*P. viridicatum*)		1
SERIE *Viridicata*	21	
P. roqueforti		18
P. aurantiogriseum (*P. commune*)		3

( ) Species classified following Pitt & Cruickshank (12).

Some strains of subgenus *Penicillium* initially identified according to Williams & Pitt (10) had to be reclassified following the latest keys (12). The most significant difference between the keys, apart from some changes in

names (*P. griseoroseum* to *P. chrysogenum*, *P. olivicolor* to *P. viridicatum*), was the reclassification of *P. aurantiogriseum*. The taxonomy of this species has been in a state of flux until very recently. It is considered today as the species named *P. cyclopium* by Raper & Thom (19). Samson et al. (20) considered this species to be a variety of *P. verrucosum* (*P. verrucosum* var. *cyclopium*). Later, Pitt (9) rejected this and accepted *P. cyclopium* as a concept but with the earlier name *P. aurantiogriseum*. However, further studies showed some confusion in Pitt's concept of this species and, currently, some species considered by Pitt to be synonyms (*P. commune* and *P. solitum*) are accepted as separate species (12). In our study, some isolates initially classified according to Pitt (9) as *P. aurantiogriseum* were reclassified following Pitt & Cruickshank (12). Three strains were classified as *P. commune* (considered the wild ancestor of the domesticated species *P. camemberti*) and seven as *P. solitum*. However, although this correction is important from a taxonomic point of view, in our opinion in a technological or even sanitary analysis of results like this the concept of *P. aurantiogriseum* accepted by Williams & Pitt (10) could be maintained because most studies on mycoflora of cheeses or on the implication of this species as spoilage agent or producer of mycotoxins pre-date the taxonomic change indicated.

Table 3 shows the occurrence of moulds in the finished product. From a practical point of view, what matters is the occurrence of the genera or species in the samples rather than the number of species identified. *Penicillium* was the dominant genus, the major species being *P. roqueforti*, *P. aurantiogriseum*, *P. verrucosum* and *P. chrysogenum*. *P. roqueforti* was dominant in the interior and clearly in the blue veins. On the surface, *P. aurantiogriseum* was the major

Table 3. Occurrence of the moulds and yeasts isolated in the finished product.

	INTERIOR SAMPLES	SURFACE SAMPLES	BLUE VEINS
Moulds (n=9):			
P. roqueforti	5(3[a])	1(1)	9(8)
P. aurantiogriseum	2	4(4)	1
P. verrucosum	4(1)	3(1)	1
P. expansum	2(1)	1	0
P. chrysogenum	1	1(1)	0
Other	2	1	0
Yeasts (n=11):			
D. hansenii	4	9(7)	ND
K. lactis	5	1	ND
Y. lipolytica	2	1	ND
Other	1	1	ND

n = total number of samples.
[a] number of samples in which the species is dominant.
ND = not determined.

species isolated. Other genera were found only in two out of nine samples from the interior and in one out of nine samples from the surface.

Penicillium was present in all the stages but mainly at the end, when it was clearly dominant. Other genera were found throughout the process, with *Cladosporium* and *Mucor* being the most significant. *Cladosporium* was found at the beginning while *Mucor* was isolated to a lesser extent at the end. Both genera are very common and widespread in nature (7) and are frequently isolated from cheese, where they are responsible for important defects (2, 4). *Cladosporium* has been found in numerous varieties, including blue cheese (21). *Mucor* has also been found both in the interior and on the surface of cheeses and it is considered one of the major contaminant fungi in cheese (2, 22, 23).

Penicillium can be considered the most frequent genus of fungi in cheeses (18, 24, 25). The species found in this study are very common in cheeses and their presence is normally undesired, for they are considered spoilage agents (2, 25). In Valdeón cheese they could be responsible for musty flavours found in some samples. Furthermore, most *Penicillium* strains associated with foods, among them those found in Valdeón cheese, are potential producers of mycotoxins, at least under experimental conditions (3). However, *P. roqueforti* is obviously desired in a blue cheese because it is responsible for the blue veins and peculiar flavour of these varieties (4).

The presence of this varied mycoflora and the dominance of some genera and species over the rest can be better understood by the different physiological properties of each fungus and the conditions of the cheese during the manufacture. The major *Penicillium* species found in Valdeón cheese are considered to be able to grow at low temperatures (min. 0-4 °C), low water activities (min. 0.83-0.85) and low pH (min. 3) (7, 25). This could explain their presence in Valdeón cheese, which is ripened under similar conditions (1). In contrast, *Cladosporium* and *Mucor* survive with difficulty to low pH's, their presence in the cheese usually being associated with insufficient acidification during the process (2). On the other hand, *P. roqueforti* is considered the *Penicillium* with lowest requirements for oxygen, which could explain its dominance over other *Penicillium* spp in cheese (7).

Table 4 shows the species of yeast identified in our study. *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Yarrowia lipolytica* and their respective imperfect forms (*Candida famata*, *C. sphaerica* and *C. lipolytica*), were the major species isolated – more than 80% of the strains identified. They were also the dominant species found in the interior of the finished cheese (Table 3). These species have been found dominant in blue cheeses and in other varieties of cheese made in a natural way. *D. hansenii* was the major species found in blue cheeses by Boer & Kuik (21), who also found *Y. lipolytica* in their samples. Devoyod & Sponem (26) found in Roquefort cheese, among other species, *Saccharomyces lactis* (now *K. lactis*), *Torulopsis famata* (now *C. famata*, anamorph of *D. hansenii*) and *Y. lipolytica*. In other varieties, *D. hansenii* and *K. lactis* were the major yeasts isolated from Saint-Nectaire cheese (27); *K. lactis*, *K. fragilis*

Table 4. Yeasts isolated and identified to species level.

Species (imperfect form)	Number of strains
Debaryomyces hansenii (*Candida famata*)	29
Kluyveromyces lactis (*C. sphaerica*)	18
Yarrowia lipolytica (*C. lipolytica*)	9
C. zeylanoides	5
C. colliculosa	3
C. parapsilosis	2
Cryptococcus laurentii	1
Rhodotorula rubra	1

and *D. hansenii* were dominant in Camembert cheese (28), while *D. hansenii* was the most important species isolated from French goat's cheeses, with *K. lactis* and *Y. lipolytica* being also present in high levels (29). Finally, *Y. lipolytica* was isolated by Schmidt & Lenoir (28) in Camembert cheese and Nahabieh & Schmidt (29) in French goat's cheese.

In the surface samples, *D. hansenii* was clearly dominant (Table 3). This agrees with the findings of other authors in other blue cheeses (30) and different varieties of cheese (27, 29).

During the manufacture, the dominance of *K. lactis* during the first stages was relevant. Devoyod & Sponem (26) observed that in another blue cheese (Roquefort) lactose-fermenting yeasts like *K. lactis* were present before the salting process, while from the salting onwards non-fermenting species developed in the cheese. According to these authors, the fermenting species, due to their gas production, could play an important role in the formation of the characteristic open texture of blue cheeses, necessary for the development of *P. roqueforti*.

Yeasts also contribute to the manufacturing process of cheeses by the neutralizing of the cheese due to the consumption of lactic acid and by their action on fat and proteins (31). Generally, fungi are considered potent proteolytic and lipolytic agents for the high production of extracellular enzymes. Although the assessment of the hydrolytic activities of the strains isolated was not the aim of this study, their relative proteolytic and lipolytic ability was determined (Table 5). The most strongly lipolytic species were *P. roqueforti*, *P. verrucosum* and *Y. lipolytica*, which produced zones of lipolysis around the colonies bigger than 3 mm. All *P. chrysogenum* strains gave a similar reaction on casein agar, used to assess the proteolytic activity, while none of the *P. roqueforti* strains produced clear zones of proteolysis. Among the yeasts, *K. lactis* showed the highest proteolytic activity.

4 Conclusions

Our results show that the mycoflora of Valdeón cheese is very diverse. Apart from *P. roqueforti*, the species desirable in a blue cheese, other species of

Table 5. Lipolytic and proteolytic activities of the major moulds and yeasts isolated.

	LIPOLYTIC				PROTEOLYTIC			
	++	+	w	–	++	+	w	–
P. roqueforti	7/9	–	2/9	–	–	–	–	9/9
P. chrysogenum	–	6/10	4/10	–	10/10	–	–	–
P. aurantiogriseum	1/5	1/5	3/5	–	3/5	2/5	–	–
P. verrucosum	8/9	1/9	–	–	2/9	3/9	2/9	2/9
Y. lipolytica	9/9	–	–	–	–	–	5/9	4/9
K. lactis	–	–	10/10	–	4/10	3/10	2/10	1/10
D. hansenii	–	1/10	8/10	1/10	–	–	–	10/10

++, strong activity (clear zone >3 mm).
+, medium activity (clear zone between 3 and 1 mm).
w, weak activity (clear zone < 1 mm).
–, no activity.

moulds and yeasts develop which may influence the characteristics of the final product. Since some of these species are usually considered undesirable in cheese because of their potential as spoilage agents and producers of mycotoxins, it seems advisable to control the hygienic and technological conditions of manufacture. Nevertheless, the possible contribution of some of these fungi to the original characteristics of this variety should not be discounted. The study of some of these strains, properly selected, will be the next step in our project.

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