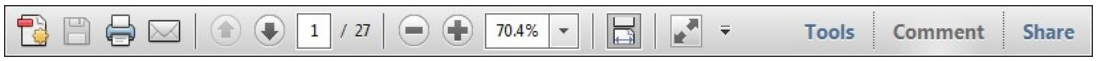
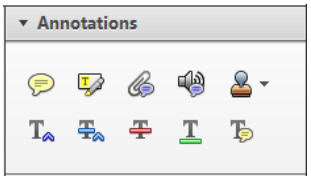
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

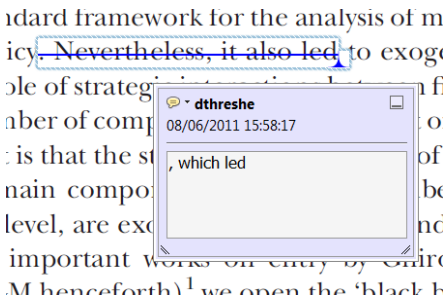


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

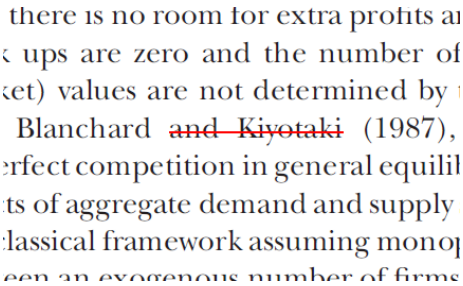


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

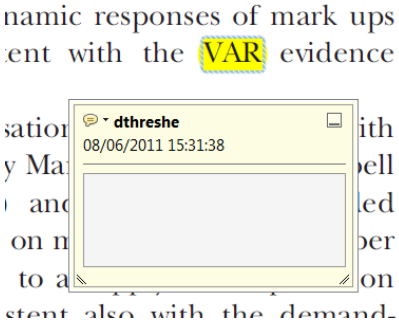


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

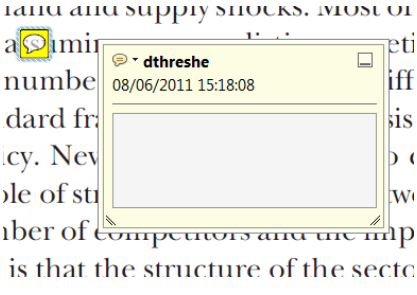


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

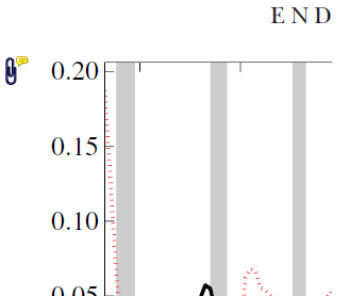


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

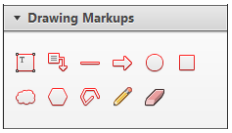
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



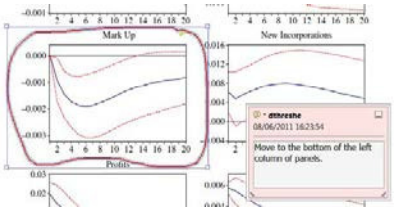
6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



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ORIGINAL RESEARCH

Occurrence of moulds associated with ovine raw milk and cheeses of the Spanish region of Castilla La Mancha

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The distribution of mould species was examined at several points of the processing chain in a Manchego cheese plant and associated dairy farms. Geotrichum *and* Fusarium *were the most frequent genera isolated in milk samples as well as in 1-month ripened cheeses, evidencing a direct transfer from raw milk. Conversely, the mycobiota of long-ripened cheeses consisted mainly of* Penicillium *species, which gained entry to the cheese through the air of ripening rooms. This study contributes to the understanding of the dynamics of fungal populations in semihard and hard cheeses, highlighting that airborne transfer from the stables could have a direct impact on their quality.*

Keywords Cheese microbiology, Dairy microbiology, DNA techniques, Raw milk.

INTRODUCTION

Cheeses have a very complex microflora that evolves through the ripening period and it is generally divided into two groups: starters and secondary flora. Starters are lactic acid bacteria whose primary function is to produce lactic acid during fermentation of lactose (Beresford *et al.* 2001). The secondary flora comprises of bacteria and fungi (yeasts and moulds) that might contribute to the development of organoleptic properties (Beresford and Williams 2004). In some cheeses, such as in blue-veined cheeses (e.g. Roquefort, Cabrales) and surface mould-ripened cheeses (e.g. Brie, Camembert), moulds are necessary to develop appearance, texture and flavour profile (Larsen and Jensen 1999; Le Dréan *et al.* 2010). Moulds might be also beneficial in other varieties of cheese where their impact on ripening is, however, not so well understood. In the cheese industry, moulds are also relevant because they can cause undesirable effects that include off-flavours, anomalous textures, discolorations and accumulation of mycotoxins (Sengun *et al.* 2008). Although the study of fungal species associated with cheese has attracted remarkable attention in the last years, few studies have, however, analysed the fungal populations in milk (Godič Torkar and Vengušt 2008; Lavoie *et al.* 2012). This information might be essential to determine whether filamentous fungi might be transferred from milk to cheese. In addition, studies on cheese mycobiota have neither taken into account the state of ripening, which might play a role in the composition of fungal populations.

Manchego is the most produced and consumed cheese in Spain. It is manufactured from locally produced ovine milk in the Spanish region of Castilla La Mancha. From a technological point of view is a semihard or hard, enzymatically coagulated, uncooked, pressed, high fat cheese (Poveda *et al.* 2003) and it is ripened at 12–15 °C and a relative humidity of 75–85% from a minimum of 30 days to a maximum of 2 years (Ruiz *et al.* 1998). Development of mould on the rind of Manchego is a common organoleptic defect and might be responsible for economic losses. In order to understand the dynamics of fungal populations associated with Manchego and to determine possible routes of contamination, we carried out an integrated study that examined the distribution of mould species at several points of the

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processing chain, including cheeses at different stages of maturation, ovine milk from associated farms, as well as brine and indoor air from the cheese plant.

MATERIAL AND METHODS

Sample collection

The samples analysed in this study were collected from a cheese processing plant in the winter of 2012 and in 20 associated farms that supply the ewe's raw milk to the plant, in the Spanish region of Castilla La Mancha. The samples of ewe's raw milk (50 mL) were collected from refrigerated tanks in the farms. The distance between farms ranged from 10 to 50 km. The samples from the plant consisted of three different batches of brine used for cheese salting and three randomly collected samples of 1, 3, 12 and 24-months ripened cheeses made with raw milk. The environmental conditions of the ripening room were 13 °C and 85% of relative humidity. All the cheeses analysed were organoleptically acceptable. The average weight of each cheese was 3 kg. In addition, the indoor air environment of the factory was also analysed as described below. All the samples were placed in sterile containers, kept at 4 °C and immediately sent to the laboratory.

Enumeration of micro-organisms in milk samples

Milk samples were serially diluted in peptone water 0.1% (w/v) (Oxoid, Madrid, Spain) and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-6}) were made, and 1 mL portions of the appropriate dilutions were pour-plated on the following media: (i) plate count agar (Oxoid, Madrid, Spain) incubated at 30 °C for 72 h for enumeration of total aerobic plate count and (ii) dichloran rose-bengal chloramphenicol (DRBC) agar (Oxoid, Madrid, Spain) incubated at 25 °C for 72 h for yeasts and moulds enumeration. DRBC plates were also used for isolation of moulds as described below.

Isolation of fungi

Filamentous fungi were isolated from milk, brine, cheeses and indoor air environment. In the case of cheeses, 25 g of rind portions randomly taken and excised aseptically was ground in a hammer mill, diluted in peptone water 0.1% (w/v) and homogenised in a stomacher for 2 min. Serial dilutions (10^{-1} to 10^{-4}) were made and 1 mL of each dilution spread as 0.1 mL aliquots over Petri dishes containing DRBC agar. The indoor air environment of drying rooms of the manufacture plant was evaluated by displaying opened agar plates containing DRBC agar for 1 h. Fungi from milk and brine were isolated from the same plates used for fungal enumeration as described above. In all cases, plates were incubated in the dark at 25 °C and colonies displaying different morphological characteristics were transferred to plates containing Sabouraud medium (Oxoid, Madrid, Spain) to obtain monosporic cultures and incubated at 25 °C for 7 day.

DNA extraction and PCR amplifications

Genomic DNA extractions of fungal isolates were undertaken using three mycelium discs excised from 5- to 7-d-old Sabouraud plate cultures and making use of the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). The ITS region was amplified by PCR using the primers and protocol described elsewhere (White *et al.* 1990). The PCR amplification of a partial region of the *EF-1α* gene (Elongation Factor 1α) was carried out in the isolates belonging to the genus *Fusarium*, with primers EF1T and EF2T (O'Donnell *et al.* 1998). PCR-amplified fragments were purified using the UltraCleanTM PCR Clean-UpTM kit (MoBio Laboratories Inc., USA) according to the manufacturer's instructions and sent for direct sequencing to STABVIDA® (Caparica, Portugal). The sequences were corrected using Chromas v 1.43 software (Brisbane, QLD, Australia) and analysed and edited using Bioedit Sequence Alignment Editor v 7.0.9.0 software (Hall 1999). Calculations of nucleotide divergence were performed using Dnastar (Lasergene, Madison, WI, USA).

Identification of fungi

Fungi were identified on the basis of the ITS sequences. *EF-1α* sequences were additionally used to verify the species identity in *Fusarium* isolates. Morphological and cultural observations were performed for isolates whose DNA sequences were uninformative. Particularly, the keys developed by Frisvad and Samson (2004) were used for *Penicillium* isolates, whereas identification of isolates belonging to genus *Aspergillus* was performed according to the keys developed by Hubka *et al.* (2013).

Statistical analysis

The Pearson correlation coefficients between variables log total counts, log number of yeasts and log number of moulds in milk samples were calculated using STATGRAPHICS CENTURION XV.II (StatPoint Technologies, Warrenton, USA).

RESULTS AND DISCUSSION

Milk can be contaminated by micro-organisms through the mammary gland, as well as during milking and storage. Once milk has been converted into cheese, micro-organisms can develop only if the environmental conditions are appropriate for their growth. Thus, numerous studies have identified and quantified bacterial and yeast populations in milk and cheeses to monitor their evolution through ripening. This approach is less applicable in the study of filamentous fungi as they are inherently more dispersible, and their spores might consequently reach the rind of cheeses at any

time of the ripening period (Ropars *et al.* 2012). Most of the reports on cheese mycobiota, however, have not taken into account the stage of maturation, an important factor that could modulate the distribution of mould species, particularly in long-ripened cheeses where physicochemical changes are normally more pronounced. As a strategy to determine potential routes of contamination, we examined the distribution of mould species at several points of the processing chain of Manchego cheese. We focused mainly on the analysis of bulk milk samples from different producers, as recent research has shown an increasing evidence that some fungal species might be transferred directly from milk to cheese (Godič Torkar and Vengušt 2008; Delavenne *et al.* 2011; Lavoie *et al.* 2012; Panelli *et al.* 2014). The results of microbial counts in ovine milk samples (Table 1) were in the range reported in previous papers that have mostly shown low levels of yeasts and moulds in raw milk (Cocolin *et al.* 2002). The correlation between total aerobic organisms and total yeasts ($r = 0.16$) or moulds ($r = 0.02$) was not statistically significant ($P > 0.05$). However, a highly statistically significant correlation was obtained between yeasts and moulds ($r = 0.89$, $P > 0.05$), suggesting that these two microbial groups could share a similar route of contamination.

Geotrichum candidum was present in 60% of the samples, being the most frequent species, with 18 isolates obtained. This yeast-like fungus is in fact generally considered a natural member of the microbiota of raw milk (Boutrou and Guéguen 2005), and its presence has been previously reported in ovine milk (Delavenne *et al.* 2011; Panelli *et al.* 2014). Some *G. candidum* isolates within the same sample displayed different cultural–morphological characteristics and were therefore selected for sequencing of ITS region. The existence of different morphotypes in this species has been already described (Missous *et al.* 2010). *G. candidum* isolates L1Y and L18A, on one hand, and L11A, L11PA and L14C, on the other hand, showed identical ITS sequences. The rest of the isolates, however, showed a high nucleotide divergence that ranged from 2.8 to 13.2%. Some studies have shown a high heterogeneity in *G. candidum* at a genomic level (Gente *et al.* 2006; Sacristán *et al.* 2013). However, the variability on the ITS sequences detected in this work could be more likely due to the existence of nonorthologous variants within this genomic region, as previously reported (Alper *et al.* 2011).

Species belonging to genera *Fusarium* (20% of positive samples), *Cladosporium* (10%), *Aspergillus* (10%) and *Penicillium* (5%) were also isolated. These genera, together with *Geotrichum*, have been commonly isolated from milk (Pešić-Mikulec *et al.* 2005; Godič Torkar and Vengušt 2008; Delavenne *et al.* 2011; Lavoie *et al.* 2012) as well as from the teat surface, silage, dust and air from farms (O'Brien *et al.* 2005; Vacheyrou *et al.* 2011), which would be therefore the most probable sources of milk contamination.

Two of the species isolated from ovine milk, *Trichosporon asahii* (5% of positive samples) and *Lichtheimia ramosa* (10%), are aetiologic agents of mastitis (González *et al.* 2001; Fadlelmula *et al.* 2009; Piancastelli *et al.* 2009). Besides, they might have also clinical relevance in humans (Shang *et al.* 2010; Woo *et al.* 2012). *T. asahii* is a yeast-like fungus (Taj-Aldeen *et al.* 2009) which is known to have caused a fatal case of septicaemia in a Guatemalan farmer with intensive exposure to sheep and goat faeces (Chan-Tack 2005). *L. ramosa,* on the other hand, is an opportunistic zygomycete fungus of the *Mucorales* order that has been recently separated from the species *Lichtheimia corymbifera* on the basis of molecular studies (Garcia-Hermoso *et al.* 2009). According to previous literature, and except for a study that showed that *L. corymbifera* was the second most frequent species in cow's milk from Quebec (Lavoie *et al.* 2012), the description of genus *Lichtheimia* in milk seems very scarce. It must be addressed that identification of members from the order *Mucorales* by standard mycological methods is very problematic, as all the species share similar morphological characteristics (Garcia-Hermoso *et al.* 2009). In addition, the name of some species from this order has been updated in recent years (Hoffmann *et al.* 2009). Therefore, until more studies are conducted, it seems difficult to accurately assess the true frequency of *Lichtheimia* species in milk.

One of the main goals of the present work was to evaluate the distribution of fungal species associated with Manchego cheeses at different stages of ripening. The species identified are shown in Table 2, and their correspondent frequencies of appearance are listed in Table 3. The results showed that whereas some species were only encountered at early stages of ripening (*Fusarium verticillioides, Fusarium oxysporum*, *G. candidum* and *Penicillium chrysogenum*), others were present in cheeses aged for either 3 to 12 months (*Penicillium roqueforti* and *Penicillium discolor*) or 12 to 24 months (*Penicillium commune*). *Cladosporium sphaerospermum* and *Penicillium solitum* were the most frequent species, being present in all the cheeses examined, except in 1-month-old cheeses. Regarding to these data, *G. candidum*, *F. verticillioides* and *F. oxysporum* might have gained entry in cheese through milk, whereas the environment of the ripening room would be the most probable origin of contamination with *Penicillium* species. In addition, brine could be a reservoir for *Penicillium commune*. A potential route for contamination with *C. sphaerospermum* could not be determined.

The differential distribution according to distinct stages of maturation would suggest that intrinsic changes occurring in cheese during ripening could have an impact on the dynamics of fungal populations, regardless of the source of contamination. Thus, physicochemical changes on the substrate could act promoting the growth of some species over others. One of the most important changes occurring during the

Table 1 Microbial counts and fungal species isolated from ovine raw milk and respective accession numbers for their ITS (nuclear ribosomal internal transcribed spacer) sequences. The same accession number was given to identical ITS sequences

Milk sample	*Total counts (cfu/mL)*	*Yeasts (cfu/mL)*	*Moulds (cfu/mL)*	*Isolate*	*Fungal species*	*Accession numbers*
1	6.6×10^3	2.4×10^2	7.4×10	L1Y	*Geotrichum candidum*	KF713522
				L1G	*Cladosporium uredinicola*	KF679757
				L1W	*Fusarium verticillioides*	KF679758
				L1093	*Aspergillus intermedius*	KF679764
2	1.6×10^4	1.8×10^3	<10	L4H	*Lichtheimia ramosa*	KF679759
3	1.1×10^4	7.5×10^2	<10	L19PC	*Geotrichum candidum*	KF713518
4	4.3×10^4	8.1×10^2	5.3×10	L19PD	*Geotrichum candidum*	KF713519
5	2.6×10^4	1.4×10^3	8×10	L5C	*Geotrichum candidum*	KF713506
				L11PA	*Geotrichum candidum*	KF713510
6	1.8×10^4	9.3×10^2	<10			
7	1.6×10^4	1.1×10^3	<10			
8	4.3×10^4	5.3×10	<10			
9	7.6×10^4	5.0×10^2	2×10	L9B	*Geotrichum candidum*	KF713507
				L9K	*Trichosporon asahii*	KF679760
10	1.6×10^4	3.1×10^1	<10			
11	6.6×10^3	5.2×10^3	2.9×10^2	L11A	*Geotrichum candidum*	KF713508
				L11B	*Geotrichum candidum*	KF713509
12	3.8×10^4	4.1×10^1	<10			
13	4.6×10^4	2.6×10^3	2.3×10^2	L13PC	*Geotrichum candidum*	KF713512
				L13095	*Fusarium verticillioides*	KF679758
				L13J	*Penicillium bovifimosum*	KF679761
14	1.6×10^4	5.1×10^3	2.4×10^2	L14C	*Geotrichum candidum*	KF713513
				L11PC	*Geotrichum candidum*	KF713511
15	3.3×10^4	8.5×10^2	<10			
16	6.6×10^5	1.1×10^2	1.9×10	L2093	*Aspergillus intermedius*	KF679764
				L16H	*Geotrichum candidum*	KF713514
				L16N	*Fusarium verticillioides*	KF679758
				L16E	*Lichtheimia ramosa*	KF679762
17	1.5×10^5	3.1×10^3	6×10	L17095	*Cladosporium macrocarpum*	KF679765
				L19PB	*Geotrichum candidum*	KF713515
18	1.3×10^5	1.1×10^3	5.1×10	L18A	*Geotrichum candidum*	KF713522
				L18I	*Fusarium oxysporum*	KF679763
19	1.5×10^6	4.8×10^3	3.1×10^2	L19C	*Geotrichum candidum*	KF713517
				L19B	*Geotrichum candidum*	KF713516
20	8.3×10^4	2.1×10^3	4.6×10	L20C	*Geotrichum candidum*	KF713521
				L20B	*Geotrichum candidum*	KF713520

cheese maturation process is the decrease on water activity (a_w), which is due to water loss and the subsequent increase on concentration of osmolytes. Recent research has revealed that optimal and limiting conditions for growth of fungi associated with cheese depend on the level of water stress, and moreover, that some fungal species respond differently if water stress is exerted by low molecular compounds derived from proteolysis, or by the salt added during manufacturing (Marín *et al.* 2014). During production of semi-hard and hard cheeses, a_w peaks at the beginning of the cheesemaking process and progressively decreases through the ripening period. In the case of Manchego cheese, average a_w at 30-days is 0.96, and it decreases until a value of 0.92 at 5 months ripening, but a further decrease to a minimum of 0.90 might occur (Ruiz *et al.* 1998). These changes on water availability could therefore have limited the growth of species sensitive to water stress, such as *G. candidum, F. oxysporum* and *F. verticillioides*. Particularly *G. candidum* is inhibited under 0.95 (Marín *et al.* 2014), while *F. oxysporum* and *F. verticillioides* are highly, if not totally, inhibited in the range of 0.90 to 0.93 (Santamarina *et al.* 2003; Jurado *et al.* 2008). These species would have been rapidly displaced by xerotolerant species from genera *Penicillium* and *Cladosporium* that might have taken advantage of the decrease on a_w after the first month of ripening (Pitt and Hocking 2009; Marín *et al.* 2014). It must be addressed, though, that Manchego, as well as other brined cheeses, has a higher concentration of salt in the rind during

Table 2 Fungal species isolated from different samples collected in the cheese processing plant and respective accession numbers for their ITS (nuclear ribosomal internal transcribed spacer) sequences. The same accession number was given to identical ITS sequences

Sample	*Number of sample*	*Isolate*	*Fungal species*	*Accession numbers*
Brine	1	BR1	*Penicillium atrovenetum*	KF679753
	2	BR2	*Peyronellaea arachidicola*	KF679754
		BR3	*Penicillium commune*	KF679767
	3	BR4	*Peyronellaea arachidicola*	KF679754
Air	1	A1	*Aspergillus niger*	KF679750
		A4	*Aspergillus niveoglaucus*	KF679751
		A5	*Cladosporium cladosporoides*	KF679752
		PCHRA	*Penicillium chrysogenum*	KF679766
		PCOM1	*Penicillium commune*	KF679767
		PDISA	*Penicillium discolor*	KF679770
		PSOL1	*Penicillium solitum*	KF679770
	2	A2	*Aspergillus niger*	KF679750
		A6	*Cladosporium cladosporoides*	KF679752
		PCHRA2	*Penicillium chrysogenum*	KF679766
		PCOM2	*Penicillium commune*	KF679767
		PDISB	*Penicillium discolor*	KF679770
		PSOL2	*Penicillium solitum*	KF679770
	3	A3	*Aspergillus niger*	KF679750
		A7	*Cladosporium cladosporoides*	KF679752
		PCOM3	*Penicillium commune*	KF679767
		PROQA	*Penicillium roqueforti*	KF679769
		PSOL3	*Penicillium solitum*	KF679770
1-month cheese	1	GC1	*Geotrichum candidum*	KF713522
		PCHR1	*Penicillium chrysogenum*	KF679766
	2	PCHR2	*Penicillium chrysogenum*	KF679766
		FV1	*Fusarium verticillioides*	KF679758
	3	FO1	*Fusarium oxysporum*	KF679763
		PCHR3	*Penicillium chrysogenum*	KF679766
3-month cheese	1	CS1	*Cladosporium sphaerospermum*	KF679755
		FV2	*Fusarium verticillioides*	KF679758
		PDIS1	*Penicillium discolor*	KF679770
		PROQ1	*Penicillium roqueforti*	KF679769
		PSOL4	*Penicillium solitum*	KF679770
	2	CS2	*Cladosporium sphaerospermum*	KF679755
		FV2	*Fusarium verticillioides*	KF679758
		PDIS2	*Penicillium discolor*	KF679770
		PROQ2	*Penicillium roqueforti*	KF679769
		PSOL5	*Penicillium solitum*	KF679770
	3	CS3	*Cladosporium sphaerospermum*	KF679755
		PDIS3	*Penicillium discolor*	KF679770
		PROQ3	*Penicillium roqueforti*	KF679769
		PSOL6	*Penicillium solitum*	KF679770
12-month cheese	1	CS4	*Cladosporium sphaerospermum*	KF679755
		PCOM4	*Penicillium commune*	KF679767
		PDIS4	*Penicillium discolor*	KF679770
		PROQ4	*Penicillium roqueforti*	KF679769
		PSOL7	*Penicillium solitum*	KF679770
	2	CS5	*Cladosporium sphaerospermum*	KF679755
		PCOM5	*Penicillium commune*	KF679767
		PDIS5	*Penicillium discolor*	KF679770

(continued)

Table 2 (Continued)

Sample	*Number of sample*	*Isolate*	*Fungal species*	*Accession numbers*
		PSOL8	*Penicillium solitum*	KF679770
	3	CS6	*Cladosporium sphaerospermum*	KF679755
		PCOM6	*Penicillium commune*	KF679758
		PDIS6	*Penicillium discolor*	KF679770
		PROQ5	*Penicillium roqueforti*	KF679769
		PSOL9	*Penicillium solitum*	KF679770
24-month cheese	1	CS7	*Cladosporium sphaerospermum*	KF679755
		PCOM7	*Penicillium commune*	KF679767
		PSOL10	*Penicillium solitum*	KF679770
	2	CS8	*Cladosporium sphaerospermum*	KF679755
		PCOM8	*Penicillium commune*	KF679767
		PSOL11	*Penicillium solitum*	KF679770
	3	CS9	*Cladosporium sphaerospermum*	KF679755
		PCOM9	*Penicillium commune*	KF679767
		PSOL12	*Penicillium solitum*	KF679770

Table 3 Frequency of isolation of fungal species (number of positive samples/number of samples analysed) from milk, cheese, brine and air samples.

	Positive samples/Total examined						
Fungal species	*Milk*	*1-month cheese*	*3-month cheese*	*12-month cheese*	*24-month cheese*	*Brine*	*Air*
Aspergillus niger							3/3
Aspergillus niveoglaucus							1/3
Aspergillus intermedius	2/20						
Cladosporium cladosporoides							3/3
Cladosporium macrocarpum	1/20						
Cladosporium sphaerospermum			3/3	3/3	3/3		
Cladosporium uredunicola	1/20						
Fusarium oxysporum	1/20	1/3					
Fusarium verticillioides	3/20	1/3	1/3				
Geotrichum candidum	12/20	1/3					
Lichteimia ramosa	2/20						
Penicillium atrovenetum						1/3	
Penicillium bovifimosum	1/20						
Penicillium chrysogenum		3/3					2/3
Penicillium commune				3/3	3/3	1/3	3/3
Penicillium discolor			3/3	3/3			2/3
Penicillium roqueforti			3/3	1/3			1/3
Penicillium solitum			3/3	3/3	3/3		3/3
Peyronellaea arachidicola						2/3	
Trichosporon asahii	1/20						

the first days after brining, before it diffuses to the core. This temporary high salinity might have been favourable for *P. chrysogenum,* a halotolerant species that grows optimally under moderate ionic stress (a_w = 0.95) (Marín *et al.* 2014).

As proteolysis advances during the ripening period, concentration of low molecular compounds increases. For example, levels of low molecular compounds measured as water soluble nitrogen are almost double after 150 days of ripening in Manchego cheese (Ruiz *et al.* 1998). This could help to explain the absence of *P. roqueforti* in cheeses ripened for more than 12 months, as this species is completely inhibited by nonionic osmotic compounds at low a_w values

(a_w = 0.925). On the contrary, *P. solitum* is a species whose growth is almost unaffected by nonionic osmotic stress in the range of 0.99 to 0.90 of a_w (Marín *et al.* 2014). The ability to grow under these conditions could therefore explain its presence in 24-month cheeses.

The distribution patterns of mould species in differently aged cheeses cannot be exclusively explained by changes on a_w. For example, *P. solitum* and *P. discolor* share very similar growth profiles in terms of osmotic stress (Marín *et al.* 2014), but the latter species was absent in long-ripened cheeses despite of being widespread in the indoor air. Similarly, the xerotolerant *Aspergillus* and *Cladosporium* species isolated from milk and the air of the ripening rooms were not detected in any of the cheeses examined. It must be addressed that although the number of cheeses examined in this study cannot be considered as exhaustive, between 50 and 200 colonies were typically screened for each sample although our results are still restricted to a single plant. Therefore, future studies considering other cheese varieties and including physico-chemical analysis of cheeses will be essential to understand the precise causes that favour the presence of certain mould species under particular environmental and substrate conditions.

Although this work has focused on the study of moulds associated with Manchego, it is possible that our results are extrapolable to other cheese varieties, as all of the *Penicillium* species identified in this work have been commonly found as spoilage agents of cheese (Lund *et al.* 1995; Kure and Skaar 2000; Kure *et al.* 2001; Ropars *et al.* 2012). In addition, it would be interesting to determine the contribution of *G. candidum* to flavour profile of Manchego, especially considering that this species was the most abundant in the ovine milk samples analysed, and its role during ripening of different varieties of cheese has been well documented (Boutrou *et al.* 2004). In the case of *Fusarium* species, some other reports have also described their presence in milk (Panelli *et al.* 2014) and cheeses with high a_w, such as unripened and smear-ripened cheeses (Montagna *et al.* 2004; Bachmann *et al.* 2005). The study of the presence of this mycotoxigenic genus deserves further studies in relation with human health, as there has not been, to our knowledge, any survey on *Fusarium* mycotoxins in cheese.

CONCLUSIONS

This study revealed that the mycobiota of indoor environment of the plant were the main source of contamination in the cheeses examined. The analysis of ovine milk destined for production of Manchego revealed a diverse fungal population that varied from farm to farm, highlighting that airborne transfer from the stables could also have a direct impact on quality of cheeses. Although water availability could play an important role by modulating the growth of some fungal species, including those that can be transferred from milk, further studies are in progress to elucidate which other key environmental variables determine the dynamics of fungal populations on cheese during ripening. This information might eventually help to identify critical control points and to design strategies to minimise spoilage of cheeses.

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