Characterisation of yeast flora isolated from an artisanal Portuguese ewes’ cheese

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Abstract

The evolution of the yeast flora was studied for an artisanal semi-hard ewes’ cheese made from raw milk. Mean log \(\text{y}^{10}\) yeast counts per gram of cheese body ranged from 2.7 to 6.4, with the higher counts observed after a ripening period of 30 days. The yeast population decreased thereafter and, at the end of curing process, reached values similar to those of the beginning. A total of 344 yeasts strains were randomly isolated from the curd and cheese body during the 60 days long ripening period. Esterase activity was common to almost all isolates (98%) while proteolysis was observed in 12% of the total yeast population. The proportion of strains with positive glucose fermentation increased from 21% in the curd and cheese body during the 60 days long ripening period to 75% at the end of the ripening period. A total of 150 isolates representative of the physiological characteristics tested were examined with the API ID 32C system showing different degrees of quality of identification. Only 15% of the strains (23 isolates) were excellently identified being assigned to the species \textit{Candida zeylanoides}. The most frequent species appeared to be \textit{Debaryomyces hansenii} (anamorph \textit{Candida famata}) and \textit{Candida intermedia}. These two species amounted to 9% of the yeasts in the curd increasing to 86% at the end of the ripening period. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cheeses; Yeast; Contamination; API system; Debaryomyces hansenii; Candida intermedia

1. Introduction

The main group of micro-organisms generally associated with cheese is composed by lactic bacteria although, nowadays, it is well recognised that yeasts isolated from cheese play a significant role in its ripening (Fleet, 1990; Deak and Beuchat, 1996). The occurrence of yeasts in cheeses may contribute positively to the flavour development during the stage of maturation or, on the contrary, may lead to product spoilage (Fleet, 1990). The recovery of yeasts in high numbers (e.g. \(10^7\)–\(10^8\) CFU/g) and their ability to hydrolyse the milk fat suggest that cheese organoleptic characteristics might be influenced by yeasts (Fleet, 1990; Deak and Beuchat, 1996). Even in cheeses inoculated with bacterial...
starters, yeasts may be detected in counts as high as 
10^3 g (Sánchez et al., 1995; Gobbetti et al., 1997).
The main defects of yeast activity include the 
production of fruity, bitter or yeasty off-flavours and 
the appearance of a gassy, open texture, being 
difficult to separate beneficial from detrimental ef-
ficts (Fleet, 1990). In addition, the defect of cheese 
surface discoloration has been recently related with 
yeast activity (Carreira et al., 1998).

The cheese studied is a semi-hard variety of ewes’ 
cheese produced in the southern region of Portugal in 
the neighbourhood of Évora city. The cheeses are 
made with raw milk without the addition of starters 
and the maturation is characterised by the predomi-
nance of lactic bacteria and enterococci (Potes and 
Marinho, 1996). The presence of yeasts was also 
observed by these authors, however a detailed study 
on this group of micro-organisms has not been 
carried out so far. The aim of this work was to 
characterise selected physiological characteristics 
and to identify the yeasts present during the ripening 
process of this artisanal ewes’ cheese.

2. Material and methods

2.1. Cheese samples

The cheese samples were collected in an artisanal 
dairy in the Évora district. The cheeses, weighing 90 
g, were produced on two different dates (April and 
May) during the same season. The ripening followed 
the usual process of this dairy (Potes and Marinho, 
1996) and cheese samples were taken after about 30, 45 and 60 days of 
maturity. A total of three to five cheeses were 
analysed at each sampling date.

2.2. Yeast enumeration, isolation and maintenance

An amount consisting of 10 g of product was 
taken from the body (inner part) of the cheeses 
without contact with the cheese rind (surface layer), 
diluted in 90 ml Ringer solution (Oxoid, Unipath 
Ltd, Basingstoke, UK) and homogenised in a blender 
(Waring Blender 700, model 31BL46, Fisher Sci-
entific, USA) for 1 min at 2000 rev./min. Serial 
dilutions were prepared and 1 ml was incorporated in 
triplicate plates of Rose Bengal (Oxoid) added 100 
ppm of chloramphenicol (Oxoid). Incubation was 
carried out over 5 days at 25°C. Counts are presented 
as average of the logarithm (log_{10}) of CFU/g of 
cheese for each sampling date. For isolations, 
colonies were randomly selected from each plate, 
according to: (i) 50% of the total colonies when the 
number of counts was between 0 and 10; (ii) 10% of 
the total colonies when their number was between 10 
and 100; and (iii) 5% of the total colonies when 
counts were between 100 and 300. Strains were 
purified by subsequent streaking onto GYP medium 
(20 g/l glucose (Merck, Darmstadt, Germany), 5 g/l 
yeast extract (Difco Laboratories, Detroit, USA), 10 
g/l peptone (Difco) and 20 g/l agar, pH 6.0) and 
maintained on slants of YM agar (3 g/l malt extract 
(Difco), 3 g/l yeast extract (Difco), 5 g/l peptone 
(Difco), 10 g/l glucose (Merck) and 20 g/l agar) at 
4°C. Fresh cultures in YM slants (24–48 h) were 
prepared before performing the tests described 
below.

2.3. Morphological characterisation

Colonies on WLN agar (60 g/l WLN (Sigma 
Chemical Co., St. Louis, USA) and 20 g/l agar) after 
4 days, at 25°C, were examined.

2.4. Physiological characterisation

2.4.1. Hydrolysis of urea

The urea hydrolysing ability was tested using 
Christensen’s urea agar (Christensen, 1946): 1 g 
peptone (Difco), 1 g glucose (Merck), 5 g sodium 
chloride (Merck), 0.012 g phenol red (MandB, 
Dagenham, UK), and 20 g agar were dissolved in 
900 ml of distilled water. The pH was adjusted to 6.8 
with 1 M NaOH. Aliquots of 4.5 ml of the medium 
were dispensed in 16 mm cotton plugged tubes and 
stirred at 121°C for 15 min. Then 0.5 ml of a 20% 
(w/v) filter sterilised (0.22 µm pore size, Millipore 
Corporation, Bedford, MA, USA) urea (Sigma 
Chemical Co., St. Louis, USA) solution was added. 
A streak of fresh culture was used to inoculate these 
agar slants and incubation was carried out at 25°C 
for 2 days. Positive tests were given by change in the 
colour of the medium from yellow to intense pink.
2.4.2. Alkalising power

Plates of a medium containing bromothymol blue (Merck) and the amino acids asparagine, L-glutamine and glycine (Carreira et al., 1998) were inoculated and incubated for 5 days at 25°C. The change in colour from yellow (pH 6.8) to blue (pH 7.6) indicated alkaline conditions caused by the yeast.

2.4.3. Form of growth in liquid medium and glucose fermentation

Tubes with GYP broth were inoculated and incubated for a maximum of 12 days at 25°C. Production of film, ring or turbidity was checked visually. Glucose fermentation was assessed by observing gas production in Durham tubes included in the GYP broth.

2.4.4. Cycloheximide resistance

 Cultures were inoculated in GYP broth containing 4 or 1000 ppm cycloheximide (Sigma). Growth was recorded after 12 days of incubation at 25°C.

2.4.5. Esterase activity

Strains were inoculated on plates of tributyrin medium (40 g/l gelatine (Difco), 24 g/l tryptone glucose extract agar (Oxoid), 5 g/l tributyrin (Sigma), 5 g/l Tween 80 (Sigma), and 10 ml/l of a solution of Nilus blue sulfate (Sigma) obtained by dissolving 66 mg of this compound in 100 ml of water) and incubated at 25°C over 4/5 days. After autoclaving the pH of this medium was 7.0±0.2. Positive results were recorded when colonies were surrounded by a transparent halo over a blue background.

2.4.6. Proteolytic activity

Strains were inoculated on plates of milk medium (250 ml of whole milk plus 500 ml of 5 g/l yeast extract (Difco), 10 g/l peptone (Difco), 20 g/l glucose (Merck) and 20 g/l agar) incubated at 25°C over 10 days. Positive results were recorded when colonies were surrounded by a transparent halo.

2.5. Yeast identification

Strains for identification were selected based on the morphological and physiological characterisation. The number of strains selected was proportional to the number of strains with similar physiological and morphological characteristics. For identification the miniaturised system API ID 32C (BioMérieux S.A., Marcy-L’Étoile, France) was used following the instructions given by the suppliers (Anonymous, 1993). Supplementary tests were performed whenever the identification was considered doubtful by the software API LAB (BioMérieux). These tests, i.e. pseudomycelium formation, nitrate assimilation, growth in tiamin and aesculin, growth at 37 and 42°C, were performed according to Kreger-van Rij (1984).

3. Results

Mean log_{10} yeast counts per gram of cheese ranged from 2.7 to 6.4, with the higher counts observed in the first production season (April) and after a ripening period of 30 days (Fig. 1). In the second production season (May) the maximum yeast population were about one order of magnitude lower after the same period. However the evolution of the yeast counts showed a similar pattern in both seasons (Fig. 1).

A total of 344 yeast strains were isolated from the curd and cheese body showing nine different morphological types (Table 1). Most strains presented the morphological types VI (61%) and V (21%). However the evolution of the different types during ripening was characterised by a decrease in the proportion of the type V and an increase in type VI. Furthermore, the pink and orange pink colonies of types VIII and IX were only isolated from the curd.

The physiological characterisation of these 344 strains is also shown in Table 1. The overall characteristics were similar in both producing seasons (April and May) and so the average results are given in Table 1. The predominant strains belong to the family Ascomycetaceae (92%, urease negative). The predominance of ascomycetous yeasts was not so high in the curd mainly because of the presence of urease positive yeasts characterised by the morphological types VIII and IX yeasts (pink and orange pink colonies). The proportion of isolates with alkalinising activity decreased from 65% in the curd to about 35% in the remaining curing period. Glucose fermentation positive strains increased from 21% in the curd to 88% and 75% after 45 and 60 days of ripening.
Fig. 1. Evolution of yeast counts (log_{10} cfu/g) in cheese body during the ripening period of artisanal ewes' cheese (production seasons: ■, April; ●, May).

Table 1
Physiological and morphological characterisation of yeasts isolated during cheese ripening period (results are indicated as percentage of positive tests and are the average of two production seasons)

<table>
<thead>
<tr>
<th>Test</th>
<th>Curd (86)</th>
<th>30 days (93)</th>
<th>45 days (90)</th>
<th>60 days (75)</th>
<th>Total (344)</th>
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<tr>
<td>Urease</td>
<td>27</td>
<td>2</td>
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<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Alkalisation</td>
<td>65</td>
<td>29</td>
<td>39</td>
<td>35</td>
<td>56</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>21</td>
<td>54</td>
<td>88</td>
<td>75</td>
<td>61</td>
</tr>
<tr>
<td>Surface growth</td>
<td>31</td>
<td>59</td>
<td>74</td>
<td>64</td>
<td>60</td>
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<tr>
<td>Esterase activity</td>
<td>99</td>
<td>98</td>
<td>97</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Proteolytic activity^b</td>
<td>14</td>
<td>21</td>
<td>5</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Cycloheximide (4 ppm)</td>
<td>74</td>
<td>81</td>
<td>93</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>Cycloheximide (1000 ppm)^b</td>
<td>26</td>
<td>47</td>
<td>20</td>
<td>12</td>
<td>27</td>
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</table>

*Morphological types^c*

<table>
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<tr>
<th>Types</th>
<th>Curd</th>
<th>30 days</th>
<th>45 days</th>
<th>60 days</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>2</td>
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<tr>
<td>II</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>3</td>
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<tr>
<td>IV</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>V</td>
<td>43</td>
<td>32</td>
<td>8</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>VI</td>
<td>22</td>
<td>60</td>
<td>81</td>
<td>88</td>
<td>61</td>
</tr>
<tr>
<td>VII</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>4</td>
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<tr>
<td>VIII</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>IX</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

^a Total number of strains isolated.
^b Total number of strains tested were 42, 38, 39 and 33 in the curd, after 30, 45 and 60 days, respectively.
^c The features edge, elevation, surface, optical and colour are as follows: I, circular with rootlike projections, rough, matte, opaque and cream; II, circular with rootlike projections, center peak, shiny, opaque and cream; III, circular with round projections, rough plane, rough matte, opaque and cream; IV, evenly circular, center peak, shiny, opaque and cream; V, evenly circular, convex, shiny, opaque and cream; VI, evenly circular, convex, matte, opaque and cream; VII, evenly circular, convex, rough matte, opaque and cream; VIII, evenly circular, convex, shiny, opaque and pink; IX, evenly circular, convex, shiny, opaque and orange pink.
ripening, respectively. The esterase activity was a feature common to almost all strains isolated (98%) during cheesemaking process. On the contrary, proteolytic activity was only detected in 12% of the isolates.

From the 344 isolated 150 were selected to be identified by the API ID 32C. The identification results are shown in Table 2. Only 15% of the strains (23 isolates) were excellently identified being assigned to the species Candida zeylanoides. For the other isolates the identifications at species level were: good (13% of the strains); doubtful (43%); and good at the genus level (5%). A total of 35 isolates (23%) did not match any of the identities given by API ID 32C.

Most strains belong to the species Debaryomyces hansenii (anamorph Candida famata), Candida intermedia and C. zeylanoides (Table 2). The difference between the species C. famata and C. intermedia was only related with formation of pseudomycelium which by the API system is considered positive for 99% of C. intermedia strains and 1% of D. hansenii/C. famata strains. Other less frequently isolated strains belong to the species Candida curvata (synonym Cryptococcus curvatus; Kurtzman and Fell, 1998) and to the genus Rhodotorula.

However, species distribution during cheese maturation was not constant. In fact, C. curvatus and Rhodotorula spp. were only recovered from the curd while the occurrence of D. hansenii/C. famata and C. intermedia increased with ripening time. In addition, C. zeylanoides was not isolated from the final stages of maturation.

The species D. hansenii/C. famata and C. intermedia showed the most frequent colony morphology of the type VI. The type V was characteristic of C. zeylanoides and C. curvata and the pink (type VIII) or orange pink (type IX) colonies were isolates of Rhodotorula spp.. However, in few isolates, the same species presented several colony morphologies which were coincident with types of other species (see Table 2).

The relation between the identification and some relevant technological properties (Table 3), revealed that esterase activity was common to all species while proteolysis was observed in 48% of the strains assigned to C. zeylanoides and was absent from D. hansenii/C. famata, C. intermedia and Rhodotorula spp.. Among the six strains of C. curvata tested only one showed proteolytic activity. The alkalizing effect was positive in 11% of C. zeylanoides, 43% of C. intermedia, 44% of D. hansenii/C. famata, 67% of C. curvata and in 90% of the Rhodotorula spp. strains. Most strains of D. hansenii/C. famata (91%) and C. intermedia (73%) were glucose fermentation positive while most C. zeylanoides were negative (84%) and all C. curvata and Rhodotorula spp. were negative.

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Curd (34)</th>
<th>30 days (32)</th>
<th>45 days (26)</th>
<th>60 days (23)</th>
<th>Total (115)</th>
<th>Quality of identification</th>
<th>Morphological type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Candida curvata</strong></td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>Doubtful (6)</td>
<td>V (5), IV (1)</td>
</tr>
<tr>
<td><strong>Candida famata</strong></td>
<td>3</td>
<td>31</td>
<td>56</td>
<td>52</td>
<td>33</td>
<td>Good (11)</td>
<td>VI (9), III (1), VII (1)</td>
</tr>
<tr>
<td><strong>Debaryomyces hansenii</strong></td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>Doubtful (27)</td>
<td>VI (25), VII (1), V (1)</td>
</tr>
<tr>
<td><strong>Candida humilica</strong></td>
<td>6</td>
<td>19</td>
<td>23</td>
<td>34</td>
<td>18</td>
<td>Good (3)</td>
<td>VI (3)</td>
</tr>
<tr>
<td><strong>Candida intermedia</strong></td>
<td>26</td>
<td>44</td>
<td>15</td>
<td>0</td>
<td>23</td>
<td>Doubtful (19)</td>
<td>VI (16), VII (2), IV (1)</td>
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<tr>
<td><strong>Candida parapsilosis</strong></td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>Doubtful (1)</td>
<td>III (1)</td>
</tr>
<tr>
<td><strong>Candida zeylanoides</strong></td>
<td>26</td>
<td>44</td>
<td>15</td>
<td>0</td>
<td>23</td>
<td>Excellent (23) Doubtful (4)</td>
<td>V (22), VI (1)</td>
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<tr>
<td><strong>Rhodotorula minuta</strong></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>Good (1)</td>
<td>IX (1)</td>
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<tr>
<td><strong>Rhodotorula glutinis</strong></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>Good (1)</td>
<td>IX (1)</td>
</tr>
<tr>
<td><strong>Rhodotorula rubra</strong></td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>Good genus (8)</td>
<td>VIII (8)</td>
</tr>
<tr>
<td><strong>Pichia carsonii</strong></td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>8</td>
<td>5</td>
<td>Doubtful (3)</td>
<td>VI (2), VII (1)</td>
</tr>
<tr>
<td><strong>Pichia etchellsii</strong></td>
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<td>0</td>
<td>0</td>
<td>3</td>
<td>Doubtful (3)</td>
<td>I (1), III (1), IX (1)</td>
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<tr>
<td><strong>Trichosporon cutaneum</strong></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>Good (3)</td>
<td>I (1), V (2)</td>
</tr>
</tbody>
</table>

The number of strains is indicated between brackets.
Table 3

<table>
<thead>
<tr>
<th>Species*</th>
<th>Esterase activity</th>
<th>Alkalising power</th>
<th>Proteolytic activity</th>
<th>Glucose fermentation</th>
<th>Lactose assimilation</th>
<th>Lactate assimilation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodotorula spp. (10)</td>
<td>100</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Candida curvata (6)</td>
<td>100</td>
<td>67</td>
<td>16</td>
<td>0</td>
<td>100</td>
<td>83</td>
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<tr>
<td>Candida zeylanoides (27)</td>
<td>96</td>
<td>11</td>
<td>48</td>
<td>26</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Candida intermedia (22)</td>
<td>95</td>
<td>43</td>
<td>0</td>
<td>73</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Candida famata</td>
<td>100</td>
<td>44</td>
<td>0</td>
<td>91</td>
<td>100</td>
<td>84</td>
</tr>
<tr>
<td>Debaryomyces Hansenii (38)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*The number of strains is indicated between brackets.

The utilisation of the API system comprised two assimilation tests (lactose and lactate) which may have significance in cheese making. Lactose positive strains represented 45, 63, 88 and 100% of the isolates in the curd, after 30, 45 and 60 days of curing, respectively. The corresponding figures for lactate positive strains were 39, 54, 68 and 85% of the yeast flora submitted to the API tests. The relation between these abilities and the species isolated is shown in Table 3. Concerning the identified strains, all C. zeylanoides, Rhodotorula rubra and Rhodotorula glutinis were lactose and lactate negative whereas the single isolate of Rhodotorula minuta was positive in both reactions. On the contrary, 100 and 83% of C. curvata strains assimilated lactose and lactate, respectively. C. intermedia assimilated lactose and lactate for 100 and 95% of the strains, respectively. Concerning D. hansenii/C. famata, the respective proportions were 100 and 84%.

4. Discussion

Yeast counts measured in the cheese at the end of the ripening period were within the range reported by other authors (Nunez et al., 1981; Chavarri et al., 1995; Fleet and Mian, 1987; Poulet et al., 1991; Litpoulou-Tzanetaki and Tzanetakis, 1992, Marcellino and Benson, 1992; Callon et al., 1994; Mor-Mur et al., 1994; Freitas et al., 1996; Hassouna et al., 1996). Similar evolution of yeast flora during ripening has also been observed and has been related with physico-chemical alterations in the cheese during ripening such as a decrease by dehydration (Nunez et al., 1981; Fleet, 1990; Macedo et al., 1993; Freitas et al., 1996). However, constant yeast counts during ripening have been reported as well (Litpoulou-Tzanetaki and Tzanetakis, 1992; Marcellino and Benson, 1992; Mor-Mur et al., 1994).

The majority of isolates belong to the Ascomycetaceae family as usually reported (see reviews of Tudor and Board, 1993; Deak and Beuchat, 1996), although a relatively higher proportion of basidiomycetous yeasts was present in the curd. Our results showing high yeast counts together with a esterase activity shared by almost all strains isolated agree with the opinion of other authors which state that yeasts may be an important microbial group determining the flavour and texture characteristics of the cheeses (Fleet, 1990; Deak and Beuchat, 1996). On the contrary, only a small proportion of strains showed proteolytic activity, as already observed by Besançon et al. (1992). In addition, we showed that proteolytic activity was preferentially present in the curd isolates and at the beginning of the ripening period. However, recently other methods for assessing the proteolytic activity, based on the breakdown of casein determined by capillary electrophoresis, seem to be more accurate to determine this activity (Clausen et al., 1997). Besançon et al. (1992) also considered that nitrate assimilation is an important technological feature of cheese yeasts but our results indicate otherwise because the strains of the most frequent genus isolated (Candida spp.) were all nitrate negative, in agreement with the respective biochemical results provided by Kurtzman and Fell (1998).

The presence of fermentative metabolism seems to be necessary to keep yeast viability in cheese body during ripening. In fact, fermentation ability was observed in 21% of the curd isolates while after 45
days of ripening this proportion increased to about
80%. These results are probably the reflect of
decreasing oxygen availability in cheese body during
maturation.

The increase in cheese pH is considered important
to cheese making because it stimulates proteolytic
bacteria activity (Fleet and Mian, 1987; Deak and
Beuchat, 1996). This pH change may be achieved by
yeast alkalisng power (Carreira et al., 1998) and by
lactate assimilation (Fleet and Mian, 1987; Deak and
Beuchat, 1996). The former characteristic was pre-
dominant at the beginning of curing because it was
common to 90% Rhodotorula spp. strains. During
ripening the percentage of strains with alkalisng
effect decreased and was kept constant due to the
presence of this feature in about 44% of the D.
hansenii/C. famata and C. intermedia strains. How-
ever, the total numbers of yeasts having alkalisng
power was similar because of the increase in yeast
population during maturation. The increase in the
numbers of isolates from these species was also
responsible for the gradual increase in the propor-
tion of lactate-positive strains during the maturation
process.

It is worth noticing that D. hansenii/C. famata and
C. intermedia were only differentiated by pseudo-
mycelium formation which is considered an unreliable characteristic for taxonomic purposes (Deak,
1991). Furthermore, the data base of the API system
refers that 1% of D. hansenii may show pseudomy-
celium while according to the data base of Deak
and Beuchat (1996) this proportion is 15%. There-
fore, it is possible that these strains of C. intermedia
are, in fact, of D. hansenii which is a far more
common contaminant of cheeses. Thus, during ripen-
ing there is only one type of dominant yeasts as these
two species represent more than 75% of the yeast
population recovered after 45 days of curing. On the
contrary, the two most frequent species of Candida
in the curd behaved differently: C. curvata was not
recovered from the cheese body and C. zeylanoides
levels decreased after 30 days of maturation (see
Table 2). This differing behaviour may be related to
the absence of fermentative ability by C. curvata
strains and with the less frequent fermentative ability
among C. zeylanoides thus limiting their growth
under the semianerobic or anaerobic conditions in
cheese body. Nevertheless, all species are able to
play a particular role in cheese ripening because they
have at least one physiological activity with tech-
nological significance, as summarised in Table 3.

Observation of colony morphology may be used as
an approximate indicator of species variability even
if either different species showed the same morphol-
y or different morphologies were observed for the
same species. In fact, the most represented colony
types were V and VI corresponding to C. zeylanoides
and D. hansenii/C. famata plus C. intermedia,
respectively (see Table 2). After 60 days of ripening
88% of the strains showed the colony type VI that
corresponded to D. hansenii/C. famata and C.
intermedia.

The identification by the API ID 32C system was
found to be quite labourious and the results obtained
were frequently doubtful. The need to use additional
tests when the quality of identification was poor
increased significantly the time and work involved.
The identification of the species described below
remains to be validated by molecular techniques
which use in microbial ecology studies has been in
constant increase (Van der Vossen and Hofstra,
1996).

The species D. hansenii/C. famata is well known
as contaminant of other cheeses (Nahabieh and
Schmidt, 1990; Besançon et al., 1992; Rohm et al.,
1992; Callon et al., 1994; López-Díaz et al., 1995;
Freitas et al., 1996; Carreira et al., 1998) and its
isolation is related with the abilities to ferment or
assimilate lactose, to assimilate lactic and citric
acids, to produce lipases and proteases and to resist
to high NaCl concentrations (Fleet and Mian, 1987).

The group of ‘pink yeasts’, like R. rubra or R.
glutinis was isolated only in the curd, being absent
during ripening. These species also have the abilities
to assimilate lactose and organic acids and to
produce lipases and proteases (Fleet and Mian, 1987)
and are normally recovered in relative low numbers
(López-Díaz et al., 1995). Their origin is related with
air contamination (Tudor and Board, 1993) and their
absence from ripened cheese body is probably due to
lower resistance to decreasing aw values and their
strict aerobic metabolism. A similar result was
reported by Freitas et al. (1995) in another type of
artisanal Portuguese cheese, where Rhodotorula spp.
represented 50% of the total counts in the beginning
of the ripening period after which they were not
recovered.

In broad terms the other species isolated during
the course of this work have already been reported in other types of cheeses. *C. intermedia* has been isolated from Camembert and Blue-veined cheeses (Roostita and Fleet, 1996) and from French goat cheese (Nahabieh and Schmidt, 1990). *C. zeylanoides* and *C. parapsilosis* were isolated from Spanish blue-cheese (López-Díaz et al., 1995). Nahabieh and Schmidt (1990) also isolated the species *C. curvata* (synonym of *Cryptococcus curvatus*, Kurtzman and Fell, 1998) which has been concerned with human or animal sources and appears to be related to the genus *Trichosporon* (Kurtzman and Fell, 1998). *Candida humicola* (synonym *Cryptococcus humicola*) is also considered to be related with the genus *Trichosporon* (Kurtzman and Fell, 1998) and is a common contaminant of cheese plants (Tudor and Board, 1993). The isolation of the species *Trichosporon cutaneum* was reported by Nahabieh and Schmidt (1990) and is usually concerned with environmental, human or animal contamination (Deak and Beuchat, 1996; Kurtzman and Fell, 1998). *Pichia etchelsii* (synonym *Debaryomyces etchelsii*) and *Pichia carsonii* (synonym *Debaryomyces carsonii*) have been isolated less frequently and reference to these species in cheeses have not been found.

The main differences from the cheese related species reported in literature concern the absence of *Kluyveromyces* spp. and *Yarrowia lipolytica*, which have been broadly isolated from cheeses (Fleet, 1990; Tudor and Board, 1993; Deak and Beuchat, 1996). *Y. lipolytica* has been isolated from radial slices (Freitas et al., 1996) or from the rind (Carreira and Loureiro, 1998; Carreira et al., 1998) of several types of artisanal Portuguese ewe's cheeses. Its absence from the cheeses analysed may be explained by its strictly aerobic growth that is not favoured under the preferential anaerobic conditions in semi-hard cheese body. These results show that a careful sampling technique must be undertaken when studying the yeast flora of cheeses because the composition of the rind and body flora are probably different.

In other Portuguese ewe’s or goat’s cheeses *Kluyveromyces* spp. was also absent (Freitas et al., 1996; Carreira et al., 1998) or was isolated in a maximum percentage of 12.5% (Macedo et al., 1995). Roostita and Fleet (1996) have observed a lower frequency of *Kluyveromyces* spp. when comparing Australian Camembert cheeses with others of French origin. Moreover, Nahabieh and Schmidt (1990) referred that the yeast flora is different in goat’s, ewe’s or cow’s cheese, stating that in goat’s cheese *Y. lipolytica* and *C. intermedia* have a significantly higher occurrence than in cow’s cheese. The use of pasteurisation does not seem to be a selective factor enhancing the occurrence of *Kluyveromyces* spp. (Nahabieh and Schmidt, 1990). Therefore, the absence of *Kluyveromyces* spp. from the cheeses studied may be related with the specificity of the respective ecological niche.

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References

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