Influence of selected factors on browning of Camembert cheese

By ALEXANDRA CARREIRA[1]*, KLAUS DILLINGER[2],
FRIEDA ELISKASES-LECHNER[2], VIRGÍLIO LOUREIRO[1],
WOLFGANG GINZINGER[2] AND HARALD ROHM[3]

[1] *Laboratório Microbiologia, Instituto Superior de Agronomia, 1349-017 Lisboa,
Portugal*
[2] *Bundesanstalt für Alpenländische Milchwirtschaft, A-6200 Jenbach, Österreich*
[3] *Institut für Milchforschung und Bakteriologie, Universität für Bodenkultur,
A-1180 Wien, Österreich*

Summary. Experimental Camembert cheeses were made to investigate the effects on browning of the following factors: inoculation with *Yarrowia lipolytica*, the use of *Penicillium candidum* strains with different proteolytic activity, the addition of tyrosine, and the addition of Mn^{2+}, thus leading to 16 different variants of cheese. Two physical colour parameters were used to describe browning, depending on the location in the cheeses: a whiteness index for the outside browning (mould mycelium), and a brownness index for the inside browning (surface of the cheese body). Mn^{2+} promoted a significant increase of browning at both locations, whereas *Yar. lipolytica* had the opposite effect. Outside browning was significantly more intense when using the *Pen. candidum* strain with higher proteolytic activity. A significant interaction was found between *Yar. lipolytica* and *Pen. candidum*. The yeast had no effect in combination with a low proteolytic strain of *Pen. candidum*, but significantly reduced proteolysis and browning in combination with a high proteolytic strain of *Pen. candidum*. We further confirmed that both strains of *Pen. candidum* were able to produce brown pigments from tyrosine, and thus both are presumably responsible for the browning activity in this type of cheese.

Keywords: Camembert cheese, browning, *Yarrowia lipoytica*, tyrosine, manganese

Cheese browning is a defect common to a wide variety of cheeses and is usually associated with products manufactured in small rural plants where the cheese-making procedures are more traditional, involving higher handling and less quality control. Although some producers claim to have losses up to 20% due to cheese browning (Nichol *et al.* 1996), the real dimension of the problem is still unknown. This is mainly due to the lack of information from the producers, who are usually reluctant in discussing their production problems. Consequently, the literature on cheese browning is scarce, particularly concerning the causes and mechanisms involved.

Asperger (1986) reported a brown-violet discoloration in Brie cheese that may

* For correspondence; e-mail: amlc@itqb.unl.pt

develop on cut surfaces and associated this phenomenon with bacteria capable of producing this coloration on cheese agar medium. The bacteria were identified as brevibacteria, micrococci and *Pseudomonas* spp., and the substrate involved was found to be tyrosine. Cheeses ripened by the mould *Penicillium roqueforti* are also prone to browning, which usually occurs either near the surface or along spike holes made to facilitate the gas exchange necessary for mould development. Based on their work with Gorgonzola-type cheeses, Nichol & Harden (1993) associated this phenomenon with the yeast species *Candida catenulata* and *Cand. lipolytica*. Weichhold *et al.* (1988) described a defect known in the German literature as 'bankrot', or shelf reddening, that occurs in many kinds of semi-hard and hard cheeses, such as Emmental, Tilsit, Provolone, Grana, Pecorino Romano and Parmesan. This defect is characterised by the appearance of a superficial brown colour that, in some cases, may intrude a few centimetres into the cheese and was associated with the growth of non-starter microorganisms such as *Lactobacillus casei* or *Pseudomonas* spp. According to Carreira *et al.* (1998), a brown discoloration of the surface of Portuguese raw ewes' milk cheese is caused by the yeast *Yarrowia lipolytica*. The involvement of this yeast species in cheese browning has also been reported for other types of cheese (Prante, 1987; Eliskases-Lechner & Ginzinger, 1999).

Although several microorganisms have been associated with cheese browning, most were found to be able to produce brown pigments from tyrosine in specific culture media (Shannon *et al.* 1977; Asperger, 1986; Weichhold *et al.* 1988; Nichol & Harden, 1993; Carreira *et al.* 1998). Therefore, it is generally assumed that the pigments result from the enzymatic oxidation of tyrosine to melanin, involving the classic Mason–Raper pathway for melanization (Mason, 1948), which is catalysed by tyrosinase. But, so far, this mechanism has never been demonstrated, and the enzymatic studies available do not confirm the existence of a tyrosinase-catalysed process in those microorganisms or in cheese. In what concerns the pathways for pigment production, only the yeast *Yar. lipolytica* has recently been studied. It was demonstrated that this is the only yeast species able to produce brown pigments from tyrosine (Carreira & Loureiro, 1998), and these pigments apparently do not result from a tyrosinase-catalysed pathway but from a disorder in the catabolic pathway of tyrosine (Carreira *et al.* 2001*a*). Formation of pigments by the yeast was also found to be promoted by Mn^{2+} (Carreira & Loureiro, 1998). Weichhold (1990) showed that bacteria are capable of metabolising tyrosine by two pathways, whereby the intermediates, homogentisic acid and 3,4-dihydroxy-phenylacetic acid play a key role. Microorganisms may lack the ring structure-degrading enzymes necessary for the degradation, leading to an accumulation of these intermediates in cheese. Under alkaline conditions, these diphenols are oxidised leading to the formation of brownish polymers.

The aim of this work was to study the effect of selected variables on Camembert browning. Several conditions presumed to favour cheese browning were included in experimental cheese-making: inoculation of *Yar. lipolytica*, *Pen. candidum* cultures with different proteolytic activity (to release different amounts of tyrosine in cheese during ripening), addition of tyrosine and addition of Mn^{2+}.

Yar. lipolytica strain and inoculum preparation

The strain used to inoculate the surface of Camembert cheeses was *Yar. lipolytica* ISA 1668 (Instituto Superior de Agronomia, P-1349 017 Lisboa, Portugal), which has already been isolated from a cheese with browning problems (Carreira *et al.* 1998) and is known to be a strong pigment producer strain (Carreira & Loureiro, 1998). A preculture was prepared by inoculating a loopful of young cells in 50 ml malt extract broth (MEB, Merck, D-64293 Darmstadt, Germany), and incubated overnight in a rotary shaker (150 rpm), at room temperature (approx. 21 °C). Ten millilitres of the reculture was then inoculated in 1 l MEB double strength, and incubated for 3 d, in a rotary shaker (150 rpm), at room temperature. The cell suspension was then centrifuged (11 300 *g*, 20 min), washed twice with physiological saline (8·5 g NaCl/l; Merck), and re-suspended in 400 ml of physiological saline in sterilised bottles with a spray system.

Cheese production

Eight different trials were carried out at the pilot plant of the Bundesanstalt für Alpenländische Milchwirtschaft (Austria), according to the standard procedure for Camembert of the stabilised type (pH 5·2 before brining, dry matter 45–48%, fat in dry matter at least 55%, size: 90 mm in diameter, approx. 250 g each). For each trial, 25 l pasteurised (74 °C, 40 s) milk was warmed to 39 °C in the vat, supplemented with 2·75 ml 3·1 M-$CaCl_2$ solution (Kisch Pharma), and inoculated with a commercial mesophilic starter, Flora Danica (Chr. Hansen A/S, DK-2970 Horsholm, Denmark), containing *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. diacetilactis* and *Leuconostoc citrovorum*, with a liquid milk culture of *Streptococcus thermophilus* (S2; Bundesanstalt für Alpenländische Milchwirtschaft, A-6200 Jenbach, Austria), as well as with *Pen. candidum*. Two strains with different proteolytic activities were used: a high proteolytic strain, Lacto-Labo® type TRAD, and a low proteolytic strain, Lacto-Labo® type F (Rhodia GmbH, D-60596 Frankfurt/Main, Germany). Four of the trials were inoculated with the strain TRAD and the other four with the strain F. Doses of *Pen. candidum* culture were calculated according to manufacturer instructions to reach a final concentration of 1×10^4 spores/ml for strain TRAD and $8·4 \times 10^3$ spores/ml for strain F. After 20–25 min of pre-ripening, at 38·5–39·5 °C, the milk was inoculated with 5 ml of commercial calf rennet (chymosin content $77 \pm 3\%$, strength 1/15000; Hundsbichler GmbH, A-6330 Kufstein, Austria). Tyrosine (Sigma Chemical Co., St. Louis, MO 63178, USA) and $MnSO_4 \cdot H_2O$ (Sigma) were also added to the milk at this stage. Preliminary experiments showed that tyrosine was not concentrated in the final cheese, and that Mn^{2+} was five times concentrated. Therefore, tyrosine was added to the milk at 1·5 mM concentration and $MnSO_4 \cdot H_2O$ at 0·2 mM. From each group of trials inoculated with one of the mould strains (four vats each), one of the vats was supplemented with both tyrosine and Mn^{2+}. Another vat was supplemented with tyrosine only, another with Mn^{2+} only, and the last was not supplemented at all. After approximately 55 min of coagulation, the curd was cut and washed with 7·5 l water. Moulding was performed 25–35 min after cutting. After 10 h at 20 °C within the moulds, the cheeses were submerged in a fresh brine (180 g NaCl/l, pH 5·2) for 90 min, and then placed in different ripening chambers. In one of the chambers, the cheeses were inoculated with the spray containing a suspension of *Yar. lipolytica*. Cheeses were sprayed at least twice in order to spread the cell suspension uniformly over the surface. Ripening conditions were similar in both

chambers: a temperature of 15–17 °C and relative humidity of 90–95 %. After 7 d all cheeses were packed (polypropylene paper, perforate 20 μm, 40 g/m^2), and ripening proceeded at a temperature of 12 °C for a further 14 d.

All production trials were prepared on one single day. A duplicate batch was produced on a different day.

Cheese analysis

Cheeses were analysed at day 1 (after inoculation with *Yar. lipolytica*), day 7 (just before packing), day 14 and day 21. At each sampling date, one cheese from each variant was used for the chemical and microbiological analysis. One independent cheese was used for the colour measurements.

Microbiological analysis

Ten grams of a cheese slice including a proportional share of the surface layer were homogenised (Stomacher, Seward Ltd., London, UK) in 90 ml of a sodium citrate solution (20 g/l), and two sets of decimal dilutions were made in quarter strength Ringer's solution.

Yeasts were enumerated on yeast extract-glucose-chloramphenicol-agar (YGCA, Merck) supplemented with 0·01 g Bromophenol blue/l (Rapp, 1974), after incubation at 25 °C for 5 d (International Dairy Federation, 1990). A solution (0·1 ml) containing 100 μg/ml of oligomycin (Merck) in ethanol (98 %) was plated on the agar surface prior to sample inoculation whenever it was necessary to prevent mould growth (Eliskases-Lechner & Prillinger, 1996). The ability of the colonies to produce brownish pigments within 24 h, at 25 °C, in the tyrosine medium, containing, per litre, 5 g yeast extract (Merck), 5 g peptone (Merck), 0·5 g glucose, 10 mM-L-tyrosine, 1 mM-MnSO$_4$·H$_2$O and 20 g agar, was used to identify the strains as belonging to the species *Yar. lipolytica* (Carreira & Loureiro, 1998).

Chemical analysis

A slice of approx. 65 g cheese including the surface layer was homogenised in a B 400 mixer (Büchi Labortechnik AG, CH-9230 Flawil, Switzerland).

Proteolysis evaluation. Proteolysis was evaluated by the *o*-phtaldialdehyde (OPA) method for both pH 4·6 soluble nitrogen (4·6-SN) and nitrogen soluble in 12 % trichloracetic acid (TS-N; Tschager, 1994). According to McSweeney and Fox (1993) both measures are indicators for the intensity of cheese proteolysis. Cheese extracts were prepared with 10 g of the ground cheese filled to 100 g with distilled water. Homogenisation was performed with an Ultra Turrax (Janke & Kunkel KG, IKA-Werk, D-79219 Staufen, Germany) for 30 s. The cheese suspension was heated for 15 min in a water bath at 65 °C, cooled in an ice bath, and the suspension was then filtered through an S&S 595 1/2 folded filter (Schleicher & Schüll, D-37586 Dassel, Germany).

For precipitation of pH 4·6-insoluble nitrogen, 5 ml cheese extract was heated to 40 °C in a water bath. One millilitre acetic acid (96 g/l) was added to the extract, which remained for a further 10 min in the water bath. After adding 1 ml sodium acetate solution (200 g/l), cooling to 20 °C and filtering the mixture (S&S 602 1/2), 1·5 ml 8 M-HCl was added to 0·5 ml filtrate. The system was hydrolysed in a drying oven at 120 °C for 12–24 h. For precipitation of 12 % trichloracetic acid (TCA) insoluble nitrogen, 2·5 ml TCA (36 g/l; Merck) was added to 5 ml cheese extract and the mixture was subsequently filtered (S&S 602h) after 10 min.

Spectrophotometric determinations. A reagent solution was prepared by adding

10 ml SDS (100 g/l; Serva, D-69115 Heidelberg, Germany) solution, 2 ml of a solution containing 2·1162 g OPA (Serva) in 55 ml ethanol (96%) and 2 ml 2-mercaptoethansulphonic acid (0·125 g/l) to 50 ml 0·1 $\text{M-Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ solution and filling to 100 ml. For the 4·6-SN estimation, 20 μl of the hydrolysed filtrate and 3 ml reagent solution were mixed in a cuvette. After 30 min, the absorption value was measured at 335 nm. The same procedure was used for the TS-N estimation, for which 20 μl were taken from the appropriate filtrate. All results are reported as g glutamic acid/kg cheese, according to the corresponding calibration curves. All spectrophotometric determinations were performed in a Lambda 20 spectrometer (Perkin Elmer, Norwalk CT 06859-0010, USA).

Tyrosine. A cheese extract was prepared with 1 g of the homogenised cheese mass, 1 ml internal standard (1 g Norvalin/l bi-distilled water; Serva) and 100 ml 0·1 $\text{M-Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ buffer, pH between 9·0 and 10·5. Homogenisation was done by 20 s mixing (Ultra Turrax). The resulting cheese suspension was then heated for 15 min in a water bath at 65 °C and further cooled in ice water. Cooled samples were filtered (S&S 595 1/2), and centrifuged (5000 $\textbf{\textit{g}}$, 20 min) in filter tubes (Ultrafree-MC, Millipore, A-1130 Vienna, Austria). The filtrate was kept frozen (-18 °C) prior to analysis. Tyrosine was determined by HPLC (Hewlett-Packard 1090 Series II, Hewlett-Packard, A-1220 Vienna, Austria) with fluorescence detection (Hewlett-Packard 1046 A).

All samples for proteolysis and tyrosine measurements were prepared in duplicate.

Colour measurements

All colour measurements were performed with a Luci100 spectral reflectometer (Dr. Lange GmbH, D-40549 Düsseldorf, Germany) equipped with a D65 xenon lamp. Six-fold measurements were made to obtain colour properties of the cheese surface. In order to access the colour of the cheese beneath the mould, the mycelium was scraped off from an area of approximately 9 cm² using a spatula. Inside measurements were also performed on 6 replicates. From the spectral reflectance data recorded in increments of 10 nm, the colour primaries were calculated and transferred into CIE-LAB coordinates L*, a* and b* by using appropriate equations (Anonymous, 1980). In order to achieve one-dimensional colour values to allow easier comparison of the samples, the Whiteness Index according to ASTM (Anonymous, 1998) was used for measurements performed on the cheese surface. For inside measurements, reflection values obtained at specific wavelengths were used to calculate a Brownness Index: $\text{BI} = 100 * (\text{R}_{590} - \text{R}_{400})/(\text{R}_{530} - \text{R}_{400})$ where R represents spectral reflectance ranging between 0 and 100%, and the indices refer to the wavelengths in nm.

Browning activity of the cultures used in Camembert production

The ability of all cultures used for Camembert production (*Pen. candidum* TRAD and F, mesophilic starter, and thermophilic starter) to produce brown pigments from tyrosine was assessed in the same tyrosine medium used to identify the yeast *Yar. lipolytica*. This medium was developed to detect brown pigment production from tyrosine and improved for the detection of *Yar. lipolytica* (Carreira & Loureiro, 1998) but, since the medium is enriched with tyrosine and is not selective, it can also be used to screen this ability in other microorganisms. *Pen. candidum* strains were pre-inoculated in 250 ml Erlenmeyer flasks containing 50 ml MEB, and incubated in an orbital shaker (150 rpm) overnight. The inoculum used for the starter cultures was the suspension used for cheese production. Of each suspension, (0·1 ml) was then plated in the tyrosine medium and incubated at 25 °C for several days.

Statistical analysis

All statistical analyses were performed by using the SAS 6·12 HP-UX software package (SAS Institute Inc., Cary, NC, USA, 1996).

RESULTS

Yeast counts

Development of yeast counts of the experimental cheeses during ripening is given in Table 1. All data are average values of two cheese productions. *Yar. lipolytica* was the dominant species in the inoculated variants where, only occasionally, non-*Yar. lipolytica* strains appeared in the lowest dilutions. Counts for non-inoculated cheeses, where yeasts were not detectable initially, correspond to non-*Yar. lipolytica* species. After 4 d of ripening, the population increased to $\approx 3{\cdot}2 \times 10^6$ cell/g, which probably resulted from a posterior contamination in the ripening chamber (data not shown). Both *Yar. lipolytica* and the yeasts on the non-inoculated cheeses grew mostly during the 1st 4 d of ripening, and the counts stabilised at day 7. Maximum level of yeasts was about ten times higher in the cheeses inoculated with *Yar. lipolytica*, and viable counts were much more stable during the last stages of maturation than in non-inoculated cheeses. Growth appeared to be similar on all variants of the inoculated cheeses although there was a slightly lower growth on the cheeses made with *Pen. candidum* TRAD than on those made with strain F. The influence of mould strain on yeast counts was much more pronounced in non-inoculated cheeses than in cheeses inoculated with *Yar. lipolytica*, especially during the last stages of ripening.

Tyrosine content

Changes in tyrosine content during ripening are also given in Table 1. Initial tyrosine content of non-supplemented cheeses was 5·2 mg/kg whereas supplemented cheeses showed values of 333 mg/kg. After 7 d of ripening, the initial levels increased in non-supplemented but decreased in supplemented cheeses. With prolonged maturation time, tyrosine increased in all cheese variants as a result of proteolysis. In general, cheeses produced with *Pen. candidum* F showed lower contents of tyrosine than cheeses with *Pen. candidum* TRAD after 21 d of ripening, and cheeses inoculated with *Yar. lipolytica* showed higher values in combination with *Pen. candidum* F but lower values with *Pen. candidum* TRAD.

Proteolysis

The increase in degree of proteolysis throughout ripening is summarised in Table 1. Cheeses with *Pen. candidum* TRAD showed a more intense proteolysis compared with *Pen. candidum* F cheeses. The influence of *Yar. lipolytica* on proteolysis was low for cheeses produced with *Pen. candidum* F. In the case of cheeses manufactured with *Pen. candidum* TRAD, proteolysis was less intense in samples inoculated with *Yar. lipolytica*. Tyrosine supplementation apparently did not influence proteolysis values. Addition of Mn^{2+} slightly delayed and repressed proteolysis in cheeses with *Pen. candidum* TRAD, but this effect was almost imperceptible in cheeses with *Pen. candidum* F.

Browning activity of Pen. candidum (*TRAD, F*), *mesophilic and thermophilic starters*

The strains of *Pen. candidum* tested were able to produce brown pigments in the medium within 2 d incubation. Intensity of the brown colour followed the same

Table 1. *Evolution of yeast counts, tyrosine, and proteolysis (4·6-SN and TS-N) during ripening of Camembert*

(Values are means for $n = 2$)

Cheese variant	Yeast counts (log cfu/g)†			Tyrosine (mg/kg)‡			4·6-SN (g glutamic acid/kg)§			TS-N (g glutamic acid/kg)§		
	Ripening time (d)			Ripening time (d)			Ripening time (d)			Ripening time (d)		
	7	14	21	7	14	21	7	14	21	7	14	21
F Y⁺ Mn⁻ T⁻	8·38	8·23	8·04	76·7	167·2	213·5	41·3	52·7	62·2	5·6	9·8	12·8
F Y⁻ Mn⁻ T⁻	7·55	7·94	7·06	47·4	108·4	165·1	39·2	44·1	57·4	5·0	7·7	11·4
F Y⁺ Mn⁺ T⁺	8·29	8·16	7·98	174·3	243·6	242·5	38·9	51·0	55·8	5·0	8·7	10·9
F Y⁻ Mn⁺ T⁺	7·52	7·49	7·15	168·1	231·0	205·6	38·9	43·5	53·9	4·9	7·2	9·9
F Y⁺ Mn⁺ T⁻	8·40	8·34	7·95	81·2	207·1	291·5	43·4	48·8	52·3	5·4	9·8	12·8
F Y⁻ Mn⁺ T⁻	7·20	7·42	7·24	42·9	132·7	213·8	38·7	46·1	52·0	4·9	8·1	11·5
F Y⁺ Mn⁻ T⁺	8·34	8·13	7·95	206·4	309·8	363·7	41·5	52·8	63·6	5·4	9·9	13·0
F Y⁻ Mn⁻ T⁺	7·54	7·32	6·94	201·6	197·3	260·4	40·1	51·6	56·9	5·2	8·9	11·5
TR Y⁺ Mn⁻ T⁻	8·18	7·87	7·91	80·5	259·2	305·4	43·7	66·7	73·2	6·1	13·6	15·8
TR Y⁻ Mn⁻ T⁻	7·44	7·67	6·27	79·5	404·6	602·2	50·2	85·2	100·9	7·9	20·0	26·1
TR Y⁺ Mn⁺ T⁺	8·16	7·86	7·95	162·9	176·3	328·9	39·3	50·5	68·5	5·2	9·7	14·7
TR Y⁻ Mn⁺ T⁺	7·23	7·20	6·62	197·9	346·6	564·4	49·7	72·6	89·5	7·8	16·0	23·0
TR Y⁺ Mn⁺ T⁻	8·22	8·18	7·97	66·8	191·1	314·0	37·9	58·0	68·3	5·0	12·2	16·2
TR Y⁻ Mn⁺ T⁻	6·91	6·73	6·45	70·6	252·9	527·4	51·0	72·7	91·7	8·1	15·1	24·6
TR Y⁺ Mn⁻ T⁺	8·04	7·92	7·75	170·1	278·2	351·4	42·0	58·5	71·2	5·9	11·8	15·2
TR Y⁻ Mn⁻ T⁺	7·02	6·65	6·20	293·9	426·2	754·1	69·6	85·2	106·4	12·2	19·9	26·9

Cheese variants: F, *Pen. candidum* F; TR, *Pen. candidum* TRAD; Y, *Yar. lipolytica*; Mn, manganese; T, tyrosine.

Superscripts ⁺ and ⁻ indicate addition, or omission, respectively.

† Yeast counts at day 1: inoculated cheeses, 3·84 log cfu/g; no yeasts were detected in cheeses without inoculation of *Yar. lipolytica*.

‡ Tyrosine content (mg/kg cheese) at day 1 in non-supplemented cheeses, 5·20; in supplemented cheeses, 333·70.

§ 4·6-SN: pH 4·6 soluble nitrogen; TS-N: soluble nitrogen in 12% trichloroacetic acid; values (in g glutamic acid/kg cheese) at day 1 of ripening in cheeses supplemented with tyrosine, 9·3 and 0·78, and not supplemented, 9·00 and 0·63, respectively.

Table 2. *Tristimulus colour values of 3-week-old Camembert cheeses*

(Values are means for $n = 2$)

Cheese variant†	Outside colour			Inside colour		
	L*	a*	b*	L*	a*	b*
F Y⁺ Mn⁻ T⁻	93·6	−0·2	5·2	79·5	0·3	18·2
F Y⁻ Mn⁻ T⁻	94·5	−0·3	5·3	78·5	1·1	18·5
F Y⁺ Mn⁺ T⁺	93·1	0·0	5·8	71·7	2·3	20·4
F Y⁻ Mn⁺ T⁺	93·2	0·0	6·1	66·0	5·6	20·8
F Y⁺ Mn⁺ T⁻	93·3	−0·1	5·5	76·5	2·0	21·4
F Y⁻ Mn⁺ T⁻	93·7	−0·1	5·8	76·6	2·2	19·6
F Y⁺ Mn⁻ T⁺	92·3	−0·2	5·3	80·4	0·3	18·1
F Y⁻ Mn⁻ T⁺	94·3	−0·3	5·1	77·8	1·6	21·3
TR Y⁺ Mn⁻ T⁻	91·4	−0·2	6·0	80·1	1·0	19·4
TR Y⁻ Mn⁻ T⁻	90·5	−0·3	10·0	82·0	1·7	19·5
TR Y⁺ Mn⁺ T⁺	89·7	−0·1	7·8	74·5	2·2	21·5
TR Y⁻ Mn⁺ T⁺	83·4	1·1	13·5	67·8	3·7	19·3
TR Y⁺ Mn⁺ T⁻	90·2	−0·1	7·2	73·1	2·4	21·4
TR Y⁻ Mn⁺ T⁻	83·5	0·9	13·7	68·6	4·2	19·8
TR Y⁺ Mn⁻ T⁺	91·6	−0·2	5·9	80·8	0·6	18·6
TR Y⁻ Mn⁻ T⁺	90·2	−0·1	11·0	79·8	2·6	21·9

† Cheese variants: F, *Pen. candidum* F; TR, *Pen. candidum* TRAD; Y, *Yar. lipolytica*; Mn, manganese; T, tyrosine.
Superscripts ⁺ and ⁻ indicate addition, or omission, respectively.

pattern of proteolytic activity: after 5 d incubation the most proteolytic strain produced a deep brown colour (TRAD strain) whereas the less proteolytic strain produced a slight reddish-brown colour only (F strain). Growth of the bacterial starters in the tyrosine medium was quite low, and no pigment production occurred.

Cheese colour

The mould layer developed homogeneously on all cheeses, irrespectively of the *Pen. candidum* culture used. After 7 d of ripening, mycelium covered the entire surface of the cheeses. Browning visibly first appeared at about 14 d. Colour properties of the cheeses, expressed in terms of CIE-LAB values, are summarised in Table 2. Brightness of the mycelium colour, which is reflected by the L*-value, ranged between 83·4 and 94·5 with a general trend towards lower brightness for cheeses produced with the TRAD mould. a*-Values, which refer to the position on the red/green-axis in the 3-dimensional colour system, were close to 0, and b*-values (position on the yellow/blue-axis) were generally positive (5·1–13·7). Cheeses produced with the TRAD mould but without inoculation with *Yar. lipolytica* showed the highest values. The span for the inside colour measured beneath the mycelium was certainly higher with L*-values ranging between 66·0 and 82·0, obviously indicating lower brightness. a* Showed measures between 0·3 and 5·6, and b*-values increased up to 21·9.

Selected examples of mature cheeses are depicted in Fig. 1. Cheeses produced with the low proteolytic mould and without any addition of Mn^{2+}, yeasts or tyrosine (Fig. 1a) showed colour values of 94·5 (L*), −0·3 (a*) and 5·3 (b*). Replacement of the standard mould with the high proteolytic variant resulted in darker cheeses (Fig. 1b) with a highly increased yellow colour component (L*, 90·5; a*, −0·3; b*, 10·0). ASTM Whiteness Indexes of these cheeses were calculated to be 58·1 and 40·8, respectively. As compared with cheeses produced with the low proteolytic mould and without any addition of Mn^{2+}, yeasts or tyrosine (Fig. 1c), a brown layer of approx.
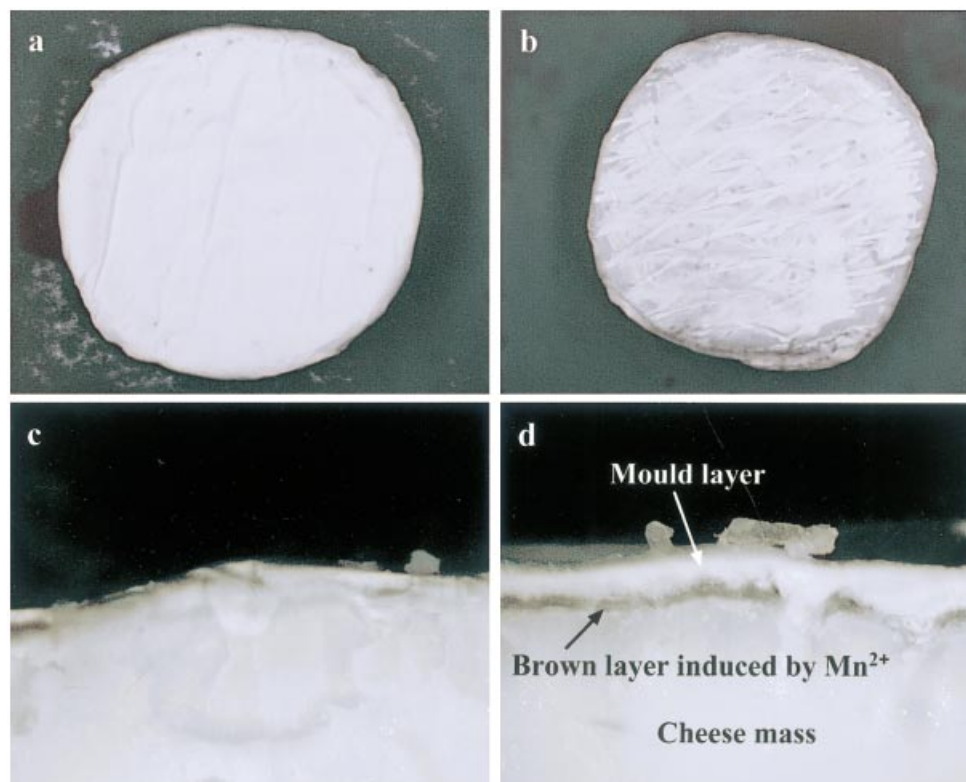
Fig 1. Appearance of Camembert produced with (*a*) a low proteolytic mould (*Penicillium candidum* F) and (*b*) a high proteolytic mould (*Pen. candidum* TRAD) without any addition of Mn^{2+}, tyrosine or yeasts. The effect of (*c*) no addition of Mn^{2+} and (*d*) addition of Mn^{2+} on the formation of a brown layer beneath the mould.

0·7 mm in thickness was found in cheeses produced with addition of manganese (Fig. 1*d*). The corresponding Browning numbers were 123·8 and 146·2 for the Mn(–) and Mn(+) cheeses, respectively.

Statistical analysis

In order to identify any systematic effects of the different cheese variants on chemical and physical measures of mature Camembert cheese (3-weeks-old), a set of analyses of variance was applied by using the General Linear Models (GLM) procedure (SAS Institute, 1996). The model comprised four main factors, i.e. addition of Mn^{2+}, tyrosine and *Yar. lipolytica*, the used mould culture and, additionally, repetition of the two separate sets of trials. Furthermore, all two-way interactions between the main effects were considered.

Table 3 summarises the mean values for each level of the main effects in terms of 4·6-SN, TS-N, tyrosine content, whiteness of the cheese mycelium, and brownness of the cheese mass. It is evident that addition of Mn^{2+} resulted in a significant decrease in cheese proteolysis, but no effect on the tyrosine content was observed. Addition of *Yar. lipolytica* also resulted in a significant decrease in proteolysis. Proteolysis was also significantly lower in cheeses with *Pen. candidum* F, which is in accordance with the higher proteolytic ability of *Pen. candidum* TRAD. Both *Yar. lipolytica* and *Pen. candidum* F induced a significant decrease in the final content of tyrosine in cheese. The outside browning of Camembert cheeses, as measured by the whiteness index,

Table 3. *Main factors influencing colour properties and proteolysis of mature* (3-*week-old*) *Camembert cheese*

(Values are means for $n = 16$)

Factor	Level	4·6-SN†	TS-N‡	Tyrosine	Outside whiteness	Inside brownness
Manganese	+	66·5*	15·4*	336	39·4*	1·37*
	−	73·8*	16·6*	376	48·1*	1·25*
Tyrosine	+	70·9	15·6	383	42·8	1·32
	−	69·3	16·4	329	44·6	1·29
Yar. lipolytica	+	64·4*	13·9*	301*	50·3*	1·26*
	−	75·9*	18·1*	411*	37·1*	1·35*
Pen. candidum	F	56·4*	11·7*	244*	55·6*	1·30
	TRAD	83·9*	30·2*	468*	31·8*	1·31
Repetition	1	71·7	17·1*	304*	40	1·33
	2	68·5	14·9*	408*	47·4	1·29

* Values in the same factor and for the same parameter, differ significantly ($P < 0.01$).

† 4·6-SN: pH 4·6 soluble nitrogen.

‡ TS-N: soluble nitrogen in 12% trichloroacetic acid.

was significantly lower in the presence of *Yar. lipolytica*, and higher in the presence of Mn^{2+} and *Pen. candidum* TRAD. Only *Yar. lipolytica* and Mn^{2+} had significant effects on the inside browning of cheese, decreasing and increasing it, respectively. A significant difference between the values of both repetitions was only observed for TS-N and tyrosine values.

In order to assess interactions between the main factors, all combinations of two factors (Mn^{2+}/tyrosine, Mn^{2+}/*Yar. lipolytica*, Mn^{2+}/*Pen. candidum* (F or TRAD), tyrosine/*Yar. lipolytica*, tyrosine/*Pen. candidum*, *Yar. lipolytica*/*Pen candidum*) were analysed using the values of all parameters of 3-week-old cheeses. Only the combination *Yar. lipolytica*/*Pen. candidum* showed a significant ($P < 0.01$) interaction for several parameters. It was observed that the presence of *Yar. lipolytica* had no influence on any of the parameters of *Pen. candidum* F cheeses. However, *Pen. candidum* TRAD cheeses showed significantly higher values of outside whiteness and lower values of proteolysis and tyrosine in the presence of *Yar. lipolytica*.

DISCUSSION

Within this multi-factorial approach to estimate the risk of cheese browning, we were able to induce Camembert browning, both on the outside of the cheeses, on the mould mycelium, as well as at the surface of the cheese paste just beneath the mycelium. The main factors presumably affecting browning are microorganisms with browning activity, tyrosine, manganese, oxygen availability and ripening stage of the cheese (Carreira *et al.* 1998; Carreira & Loureiro, 1998; Weichhold, 1990).

Pen. candidum was found to be able to produce brown pigments in a culture medium containing tyrosine and Mn^{2+}, and this activity was more intense for a more proteolytic strain. This is in agreement with the correlation between browning of cheeses and proteolysis, as higher proteolytic values (4·6-SN values > 85 g glutamic acid/kg and TS-N values > 20 g glutamic acid/kg), reached in cheeses with *Pen. candidum* TRAD only, were always associated with deeper brown colours. This higher proteolysis obviously resulted in higher concentrations of tyrosine in cheese (contents > 500 mg/kg). Therefore, it can be assumed that a high proteolytic activity is needed to achieve browning. Increased concentrations of tyrosine were already associated with browning of Australian Gorgonzola cheeses, and this was

correlated to an excessive humidity in the ripening chambers that resulted in an increased proteolysis (Nichol & Harden, 1996). Although tyrosine is one of the main factors of cheese browning, supplementation had no influence. Supplemented tyrosine was metabolised at the beginning of ripening and its final concentration, however, was only influenced by proteolysis.

Nevertheless, proteolysis of *Pen. candidum* is influenced by interactions with the competing microflora in cheeses (Gripon, 1993). Cheeses with *Pen. candidum* TRAD inoculated with *Yar. lipolytica* showed less browning and the tyrosine contents were approx. 50% lower compared with the uninoculated cheeses, indicating that *Yar. lipolytica* suppressed proteolysis. Both inside and outside browning were significantly reduced in the presence of *Yar. lipolytica*. These findings are controversial according to the assumption in the literature that *Yar. lipolytica* contributes to cheese browning, as this yeast was isolated from several cheeses with this defect (Nichol & Harden, 1993; Carreira *et al.* 1998; Eliskases-Lechner & Ginzinger, 1999) and produces brown pigments from tyrosine through a process strongly stimulated by Mn^{2+} (Carreira & Loureiro, 1998). A possible explanation is that lower proteolysis results in lower tyrosine contents in the presence of *Yar. lipolytica* in cheese.

Mn^{2+} is known to promote the production of brown pigments from tyrosine in culture medium (Carreira & Loureiro, 1998), by catalysing the oxidative conversion of diphenols into brown pigments (Carreira *et al.* 2001*b*). Mn^{2+} also promoted the formation of brown pigments on the cheese surface and the cheese paste just beneath the mycelium. This influence of manganese could explain the sporadic appearance of browning problems in cheese due to seasonal variations of the manganese content in milk (Monnet *et al.* 1995).

Based on the findings of this study, cheese browning as a multi-factorial process depends on the intensity of proteolysis, interactions of the microflora, and the content of manganese. High proteolysis resulted in a high tyrosine content, which has been described as a substrate for brown pigments (Carreira & Loureiro, 1998; Weichhold, 1990). The occurrence of the brown pigments only on the surface indicates that an oxidation reaction is involved in the browning of Camembert cheese. Besides the proteolytic activity, the growth rate and density of the *Pen. candidum* culture and the height of the mycelium are important factors for controlling appearance and discoloration defects of cheese.

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