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Proteolysis in model Portuguese cheeses: Effects of rennet and starter culture

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Abstract

To shed further light onto the mechanisms of proteolysis that prevail throughout ripening of Portuguese cheeses, model cheeses were manufactured from bovine milk, following as much as possible traditional manufacture practices – using either animal or plant rennet. The individual role upon proteolysis of two (wild) strains of lactic acid bacteria – viz. *Lactococcus lactis* and *Lactobacillus brevis*, which are normally found to high viable numbers in said cheeses, was also considered, either as single or mixed cultures. Our experimental results confirmed the influence of rennet on the proteolysis extent, but not on proteolysis depth. On the other hand, the aforementioned strains clearly improved release of medium- and small-sized peptides, and contributed as well to the free amino acid pool in cheese.

Keywords: Cheese ripening; Soluble nitrogen fractions; Casein hydrolysis; Cheese coagulant; Lactic acid bacteria

1. Introduction

Proteolysis has for long been regarded as the most important multi-step, biochemical event in cheese ripening (Fox, Law, McSweeney, & Wallace, 1993; Fox & McSweeney, 1996); in fact, it accounts for the development of a number of organoleptic features, encompassing both flavour and texture. The main vectors for proteolysis in cheese are proteinases indigenous to milk (plasmin and cathepsin D), included in the coagulant formulation (either from animal, plant or microbial origin), and released upon lysis by starter and non-starter bacteria. Each one plays a specific role, but the whole set, rather than each one individually, affects the final product.

Portuguese cheeses bearing DOP status are traditionally manufactured from whole raw milk; the most usual cheesemaking protocols encompass milking of animals by early morning, brief storage of milk at room temperature, clotting with plant rennet in the absence of any added starter culture, cutting and manual working of the curd, spontaneous whey drainage, and ripening for a minimum of 30 days (Macedo, Malcata, & Oliveira, 1993). A broad group of microorganisms eventually gain access to the curd from the cheesemaking environment, and will consequently contribute to the ripening process. Lactic acid bacteria are largely predominant in such a microbial group, and are believed to play a key role on the organoleptic characteristics of the final cheese. However, studies pertaining to model cheeses under strictly controlled conditions are scarce to date, so knowledge on the specific effect of each factor upon the final cheese is still poor.

Clotting of milk is usually an enzyme-driven step during which rennet enzymes bring about cleavage of Met_{105}–Phe_{106} of κ-casein, that is present on the surface of casein micelles in milk, surrounding α_s- and β-caseins (which are more hydrophobic in nature). Animal rennet possesses a pair of enzymes: chymosin and pepsin. Plant rennet (viz. that extracted from dried flowers of the wild thistle, *Cynara cardunculus* L.), which has been used for centuries in the manufacture of traditional cheeses in Portugal (Freitas & Malcata,

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2000; Macedo et al., 1993; Sousa & Malcata, 1997a), also contains two enzymes – cardosin A and cardosin B (Veríssimo et al., 1996); the former is somewhat similar to chymosin, whereas cardosin B is a pepsin-like enzyme (Ramalho-Santos, Veríssimo, Faro, & Pires, 1996). These similarities may explain why that plant rennet performs so well in cheesemaking, even though cardosin A cleaves more peptide bonds than chymosin does (Macedo, Faro, & Pires, 1996; Sousa & Malcata, 1998), hence generating dissimilar patterns of proteolysis, which account for the differences found between cheeses (Sousa, Ardö, & McSweeney, 2001).

One major problem of cheesemaking with raw milk, as is the case of traditional Portuguese cheeses, is the adventitious microflora present, coupled with the lack of pasteurization of milk and sterilization of manufacturing tools that will eventually permit inclusion of undesirable microorganisms, all of which will undergo different evolution patterns throughout ripening. Such population dynamics is partially responsible for several important differences between cheeses, and for unique characteristics of Portuguese traditional cheeses. Hence, study of native bacteria normally found in traditional cheeses, coupled with full understanding of their role in controlling the final characteristics of those products, appears essential, either individually or when undergoing interactions with other constituents of the microflora.

In attempts to characterize the effect of selected biotechnological parameters upon proteolysis in Portuguese traditional cheeses, model cheeses were therefore experimentally produced on a laboratory scale, following as much as possible traditional manufacture practices. Rennet enzymes and adventitious starter microorganisms were specifically assayed for their effects on cheese primary and secondary proteolysis, both of which are responsible for a major part the specific flavour and texture characteristics of such gourmet food specialities (Fernández-Salguero & Sanjuán, 1999; Freitas & Malcata, 1996).

2. Materials and methods

2.1. Manufacture

Model cheeses were manufactured from standardized (3%(w/v) fat), microfiltered, autoclaved bovine milk, supplied by Escola Superior Agrária de Coimbra (Portugal). Two alternative commercial rennets were used to effect milk coagulation – animal rennet (LusoCoalho, Portugal) and plant rennet (Formulab, Portugal).

Eight 2-l batches of milk were processed, according to a full factorial design replicated twice, encompassing two types of rennet from animal and plant origin, and four types of microbial inoculum, using wild strains of *Lactobacillus brevis* (LMG 6906) and *Lactococcus lactis* (LMG S 19870), both from the collection currently held by Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit (Gent, Belgium), independently and as a 1:1 coculture (using also absence of inoculum as control). The manufacture protocol followed traditional Portuguese practices (Macedo et al., 1993), except that the whole process was carried out in a sterile flow chamber using sterile tools and equipments. Following inoculation of each batch of milk as appropriate, 0.22 μm filter-sterilized (Orange Scientific, Belgium) animal or plant rennet was added with equivalent clotting powers. The milk was then distributed into 100 ml-sterile flasks, with a diameter of 5 cm. The mixture was incubated for 1 h at 30 °C to allow clotting; afterwards, perpendicular vertical cuts were made in the curd, followed by incubation for an extra 90 min. The curd was then slightly compressed, in order to aid in whey removal. The process was finalized by brining the experimental cheeses for 5 min, in a (sterile) saturated sodium chloride aqueous solution (330 g l^{-1}). Upon removal, cheeses were ripened in sealed flasks up to 60 d at 7 °C.

2.2. Physicochemical characterization

Model cheeses were assayed, throughout the ripening period, for moisture content by oven drying (IDF, 1982), and for total protein content by Kjeldahl (IDF, 1985) using a dedicated Kjeltec System, composed of a 2012 Digestor and a 1002 Distilling Unit (Tecator, Sweden). The pH of cheeses was directly measured with a pH meter (Micro pH 2002, Crison, Spain).

2.3. Proteolysis assessment

Cheeses picked at random at 1, 7, 21, 35, 49 and 60 d of ripening were sampled, and samples were assayed for proteolysis. Nitrogen soluble in water, WSN, and nitrogen soluble in 12%(w/v) trichloroacetic acid (Merck, Germany), TCASN, were determined following extraction, as described by Kuchroo and Fox (1982), except that a Sorvall Omni-mixer (Dupont, USA) was used for homogenization, and that the supernatant obtained was filtered through No. 42 filter paper (Whatman, UK). Nitrogen soluble in 5%(w/v) phosphotungstic acid (Merck), PTASN, was also determined by Kjeldahl, according to Stadhouders (1960), except that extracts were prepared as described above. Using the experimental data generated, three proteolytic indices were calculated as ratios between the various soluble fractions prepared to total nitrogen, TN: ripening extension index, WSN/TN; ripening depth index, TCASN/TN; and free amino acid index, PTASN/TN.

Water soluble extracts (WSE) and water insoluble extracts (WISE) were obtained from the aforementioned cheese samples, and duly lyophilized (HetoDrywinner, Denmark); then, 7 mg of WISE and 25 mg of WSE were mixed with sample buffer (pH 7.6), containing 0.12 M tris(hydroxymethyl)aminomethane (Merck), Tris, 8.2 M urea (Merck), 2.5 mM EDTA (Pronalab, Portugal), 0.2 M β-mercaptoethanol (Merck) and 0.01%(v/v) bromophenol blue (Merck), stirred and centrifuged (Universal 32 R, Hettich, Germany) at 10,000 rpm, for 10 min at 4 °C. A 15-μl aliquot of the supernatant and a 12-μl aliquot

of bovine casein fractions were applied into the wells of electrophoresis plates.

Polyacrylamide gel electrophoresis using urea (urea-PAGE) of samples was then performed, according to Andrews (1983) with modifications. The assays were carried out in vertical plates (Bio-Rad, USA), using a Power-Pac Universal power supply (Bio-Rad). The slab gels consisted of 4.2%(w/w) stacking gel and 12.5%(w/w) running gel; the former gel buffer was 0.06 M Tris and 4.5 M urea (Merck) (pH 7.6); and the latter gel buffer was 0.76 M Tris and 9 M urea (Merck) (pH 8.9). The electrophoresis buffer was 0.02 M Tris and 0.19 M glycine (Merck) (pH 8.4). Running was performed at 20 °C, at 280 V until the end of the stacking gel was reached, and at 300 V afterwards. The gels were stained directly by the method of Blakesley and Boezi (1977) with Coomassie Brillant Blue G-250 (Merck), and destained using distilled water. The gels were finally digitized (GS-700 Imaging Densitometer, Bio-Rad) and scanned so as to quantify bands by densitometry (Molecular Analyst v. 1.4 software, Bio-Rad).

2.4. Statistical analyses

All experimental results (encompassing the various soluble nitrogen fractions throughout ripening time) were analysed using SPSS v. 14.0.0 software (SPSS, USA) to assess statistically significant differences (at the 5% level of significance) between the four types of inoculum and the two types of rennet; non-parametric tests were employed, because the original experimental data failed to be normally distributed.

3. Results and discussion

3.1. Physicochemistry

Microfiltered milk and model chesses were evaluated for total protein and moisture contents, as well as pH changes; the results of such a characterization are shown in Table 1. From inspection of these data, one finds that the type of inoculum had no significant impact on the levels of protein content and moisture. On the other hand, significant differences concerning total protein were found between model cheeses made with animal or plant rennet; nevertheless, only plant rennet exerted influence on the moisture content of model cheeses, between 0 and 60 d of ripening. Note that the somehow erratic behaviour of data on protein content may have resulted from use of independent cheese replicates, with each cheese sampled only once throughout the entire experimental program. Data pertaining to pH indicate the influence of inoculum upon acidification of model cheeses. The two strains used (viz. *L. lactis* and *L. brevis*) showed statistically different behaviours upon acidification as single cultures; in the mixture, the pH profile was similar to that obtained for *L. lactis* alone, thus indicating its higher acidification capacity (statistical data not shown).

3.2. Proteolysis

3.2.1. General

The contribution of both rennet and starter bacterium upon proteolysis was estimated quantitatively via calculation of the aforementioned three ripening indices, and qualitatively by electrophoresis. The evolution of these indices, throughout ripening of model cheeses, is depicted in Fig. 1. The electrophoretic patterns of the water-insoluble and -soluble extracts of cheese are depicted in Fig. 2.

3.2.2. Primary proteolysis and ripening extension index

This index reflects the amount of casein-derived peptides, which are mainly a result of the direct action of residual rennet, retained in the curd after manufacture, on that substrate protein. The products in this fraction range from large peptides through a range of medium sized peptides.

The evolution of WSN, for both types of rennet and all four types of inocula, exhibited three distinct stages (Fig. 1a); a significant increase ($P < 0.05$) from 1 to 7 d of storage; a hardly significant increment ($P > 0.05$) until 35 d; and a significant increase towards the last week of rip-

Table 1
Bulk physicochemical characterization of milk and model cheeses, concerning the various types of inoculum and of rennet tested[a]

		Processing parameter					
		Rennet		Inoculum			
		Animal rennet	Plant rennet	Control	*L. lactis*	*L. brevis*	Mixed culture
Total protein %(w/w$_{dry\ matter}$)	Milk	28.8 ± 5.1^{b}					
	0-d cheese	55.4 ± 13.1^{c}	53 ± 19^{c}	59 ± 10^{b}	63.4 ± 5.0^{b}	63.5 ± 5.0^{b}	43 ± 21^{b}
	60-d cheese	27.7 ± 5.3^{b}	$32.4 \pm 9.4^{b,c}$	31 ± 13^{b}	28.8 ± 6.4^{b}	27.8 ± 4.6^{b}	32.4 ± 6.4^{b}
Moisture %(w/w)	Milk	91.7 ± 1.1^{b}					
	0-d cheese	84.1 ± 2.9^{c}	86.60 ± 0.90^{c}	85.9 ± 1.6^{b}	83.2 ± 4.1^{b}	86.6 ± 1.4^{b}	85.70 ± 0.60^{b}
	60-d cheese	82.3 ± 4.0^{c}	84.3 ± 2.0^{d}	82.2 ± 4.0^{b}	85.5 ± 2.8^{b}	82.8 ± 3.9^{b}	82.4 ± 1.9^{b}
pH	Milk	6.72 ± 0.03^{b}					
	0-d cheese	6.18 ± 0.03^{c}	6.14 ± 0.03^{c}	6.18 ± 0.05^{c}	6.18 ± 0.04^{c}	6.13 ± 0.03^{c}	6.15 ± 0.01^{c}
	60-d cheese	4.70 ± 0.80^{d}	4.65 ± 0.95^{d}	6.10 ± 0.13^{c}	4.27 ± 0.12^{d}	4.30 ± 0.18^{d}	4.01 ± 0.07^{d}

[a] Results expressed as mean ± standard deviation.
[b,c,d] Means for each physicochemical parameter, within the same column without a common superscript, are significantly different ($P < 0.05$).

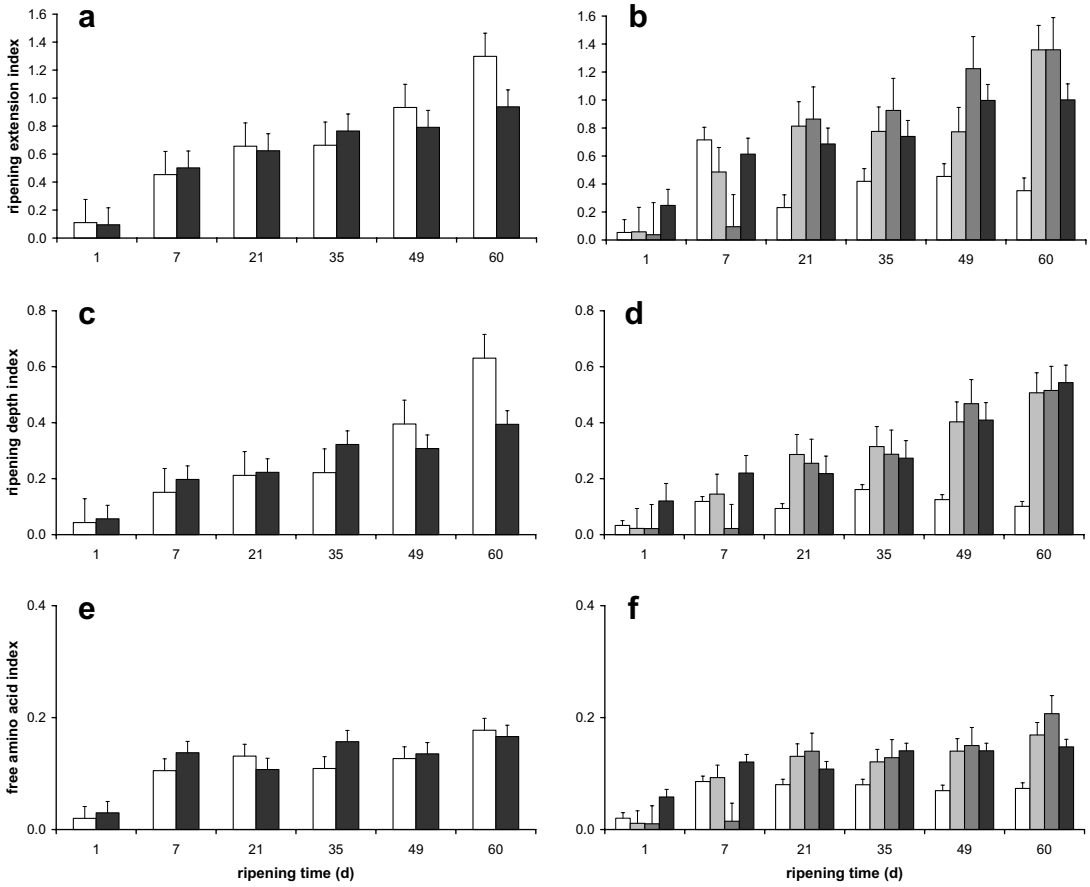

Fig. 1. Evolution with time of proteolysis indices of model cheeses: (a) effect of animal rennet (□) and plant rennet (■) on the ripening extension index; (b) effect of none (□), *Lactococcus lactis* (▒), *Lactobacillus brevis* (▓) and mixture of both (■) on the ripening extension index; (c) effect of animal rennet (□) and plant rennet (■) on the ripening depth index; (d) effect of none (□), *L. lactis* (▒), *L. brevis* (▓) and mixture of both (■) on the ripening depth index; (e) effect of animal rennet (□) and plant rennet (■) on the free amino acid index; and (f) effect of none (□), *L. lactis* (▒), *L. brevis* (▓) and mixture of both (■) on the free amino acids index. Error bars represent the standard error of the corresponding means.

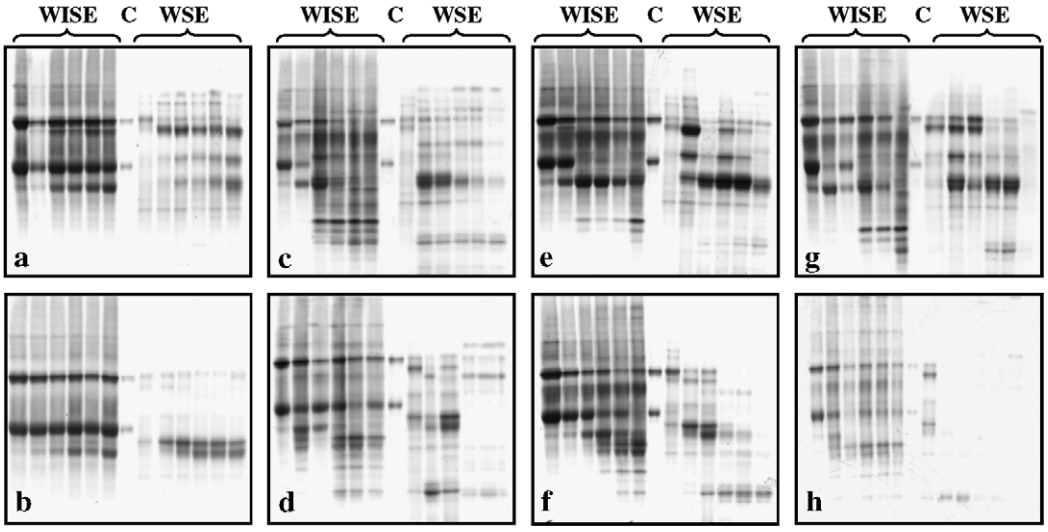

Fig. 2. Evolution with time of urea-PAGE electophoretograms of water-insoluble extracts (WISE) and water-soluble extracts (WSE) of model cheeses by 1, 7, 21, 35, 49 and 60 d(1st through 6th lane, respectively): effect of (a) animal rennet and (b) plant rennet, in the absence of inoculum; effect of (c) animal rennet and (d) plant rennet, in the presence of *Lactococcus lactis*; effect of (e) animal rennet and (f) plant rennet, in the presence of *Lactobacillus brevis*; and effect of (g) animal rennet and (h) plant rennet, in the presence of a mixture of both. C: bovine casein standard.

ening ($P < 0.05$). These pieces of evidence suggest an initial gradual effect of residual clotting enzymes, until they become inactivated and their substrates are depleted as time elapses (O'Keefe, Fox, & Daly, 1976). Milk proteinases could not contribute to this primary proteolysis, because they had been inactivated when milk was thermally treated prior to manufacture. The increment during the later stage of ripening is associated with bacterial proteinases, which are extensively released via lysis of their source microflora.

By inspection of Fig. 1a, one observes that WSN/TN levels increased from 0.09–0.11 in the curd to 0.9–1.3 by 60 d of ripening; no significant differences ($P > 0.05$) throughout ripening were found between the two types of rennet employed. These results do not (surprisingly) agree with the findings by Nuñez, Fernandéz del Pozo, Rodríguez-Marin, and Pires (1991), or Sousa and Malcata (1997b) for that matter, pertaining to La Serena and Serra da Estrela cheeses, respectively, who reported significantly higher values for plant-coagulated cheeses; however, note that bovine, rather than ovine milk, was employed in our case.

The effect of inoculum upon primary proteolysis presented a great variability (Fig. 1b); the values ranged from 0.04–0.25 in the curd to 0.35–1.36 by 60 d. The larger discrepancies, especially by the end of ripening, were found mainly between control model cheeses, and those inoculated with any form of starter culture. In the final week, however, significantly higher WSN/TN values for model cheeses inoculated with *L. brevis* and *L. lactis* were apparent, when compared to the other model cheeses – and especially with control cheeses. The presence of inoculum did not essentially affect the WSN/TN values during the first 2–3 weeks, which is in agreement with the well-accepted role of residual rennet upon casein initial breakdown.

In general, the electrophoretic trends observed are consistent with those of WSN/TN – with degradation products denoted as bands with higher mobility than the original caseins; in control cheeses, the electrophoretic pattern is much simpler (Fig. 2a and b), because there are no bacteria to release proteinases able to further breakdown the initial polypeptides. Degradation of α_s-casein leads indeed to appearance of bands with higher electrophoretic mobilities, *viz.* α_s-I-casein and smaller peptides. Bands accounted for by hydrolysis products of α_s-casein showed a progressive decrease in intensity, and by 60 d of ripening the original band had vanished in all model cheeses (Fig. 2c–h). Differences in the molecular weight distribution of peptides produced by the two coagulants are also visible: some of the pre-α_s-casein bands produced by plant rennet exhibited lower electrophoretic mobilities than those produced by animal rennet. Such a trend was observed by Freitas and Malcata (1996) in Picante cheese, as well as by Sousa and Malcata (1997b) and by Fernández del Pozo, Gaya, Medina, Rodriguez-Martín, and Nuñez (1988) in ovine cheeses.

In all cases, β-casein was hydrolysed to a much lesser extent than α_s-casein, which is in agreement with reports by other authors (Awad, 2006; Kim & Jimenez-Flores, 1994; Sousa & Malcata, 1997a; Veloso, Teixeira, Peres, Mendonça, & Ferreira, 2004). According to these authors, the more hydrophobic nature of β- than α_s-casein may promote preferential binding of the former to milk fat globules, thus protecting it from proteolytic attack. Degradation products derived from β-casein account for bands with slower mobility, classically designated as γ-casein (Creamer, 1975; Fox et al., 1993; Grufferty & Fox, 1988a, 1988b). Furthermore, a peptide with a slightly greater mobility than β-casein (i.e. β-I-casein) was reported as associated with the action of chymosin or with proteases from *C. cardunculus* over Leu_{192}–Tyr_{193} or Ala_{189}-Phe_{190} in bovine β-casein (Sousa & Malcata, 1997a, b).

The WSE electrophoretic patterns were characterized by an increasing number of bands as ripening time elapsed, which is consistent with the increase in WSN/TN values. Furthermore, visible differences were found in the electrophoretograms between inoculated (Fig 2c–h) and control cheeses (Fig. 2a and b).

Regardless of the effect of rennet and bacterial proteinases on proteolysis in bovine model cheeses, it is important to stress that our study was carried out using microfiltered milk subjected to thermal treatment; hence, a few changes may have resulted, including deactivation of indigenous enzymes, coupled with slight denaturation of whey proteins and disruption of their original interaction with caseins, all of which will likely influence activity and accessibility of casein to proteinases (Grappin & Beuvier, 1997; Lau, Barbano, & Rasmussen, 1991; McSweeney, Fox, Lucey, Jordan, & Cogan, 1993); hence, extrapolation to actual cheeses is to be done with care. However, the alternative to mimic actual cheeses more closely would have been to use unprocessed raw milk and rennet – which would contain too many uncontrolled variables, and hence too many sources of variability that would hamper rationalization of each biotechnological effect upon the final cheese matrix.

3.2.3. Secondary proteolysis and ripening depth index

This index correlates with the amount of intermediate- and small-sized peptides (2 to 20 amino acid residues). Considering both the effects of type of rennet and type of inoculum, the TCASN/TN ratio increased in value progressively with time, in a fashion similar to that of WSN/TN until the end of the ripening period; however, from a statistical point of view, there was no intermediate plateau as observed previously for WSN/TN (Fig. 1c–d).

When considering the influence of the source of rennet on this index, significant differences ($P < 0.05$) were found only by 35 d; by this time, model cheeses manufactured with plant rennet possessed more intermediate- and small-sized peptides than their animal rennet counterparts.

In terms of the effect of inoculum on evolution of ripening depth index, the same trend held as observed for the ripening extension index: the values of control model cheeses were statistically different from their inoculated counterparts only from 49 d onwards. Among the

inoculated model cheeses, significant differences were found only at the start of ripening (viz. 1 to 7 d), whereas during the remaining period either starter bacteria (*L. lactis*, *L. brevis* or a mixture thereof) exhibited similar behaviours ($P > 0.05$); the action of enzymes released upon cell lysis led in all cases to formation of small peptides and free amino acids (O'Keefe et al., 1976). Control model cheeses remained essentially constant in their profile, thus suggesting that rennet was no longer active towards further degradation of smaller peptides, and that the starter culture(s) played an active role upon such degradation.

3.2.4. Secondary proteolysis and free amino acid index

This index accounts for the actual presence of amino acids in free form, the final product of proteolysis, as well as the smallest peptides, i.e. those containing less than 6 amino acid residues (Grappin & Beuvier, 1997).

There were no significant differences throughout ripening between cheeses manufactured with rennet from either source. Their levels increased, especially during the first week of ripening ($P < 0.05$), with further increments being much less significant (Fig. 1e). Control model cheeses did not exhibit significant variation in this index throughout ripening ($P > 0.05$), except again during the first week, which coincides with the trend observed for WSN/TN values. Therefore, rennet does not seem to play any significant role, unlike enzymes from the starter culture(s). In fact, statistically significant differences ($P < 0.05$) between control model cheeses and their inoculated counterparts were attained by 35, 49 and 60 d of ripening, for cheeses inoculated with the mixed culture, and with *L. lactis* and with *L. brevis*, respectively. By 7 d, *L. brevis* led to the lowest values for this index ($P < 0.05$), when compared to the remaining model cheeses inoculated. In contrast, *L. lactis,* either as a single or as a mixed culture, had a greater capacity to release free amino acids. This bacterium is well-known for its proteolytic system, which includes a cell-wall proteinase able to hydrolyse caseins into smaller peptides, and a set of peptidases that can further degrade these peptides into free amino acids (Bourdat-Deschamps, Le Bars, Yvon, & Chapot-Chartier, 2004; Pillidge et al., 2002).

Among inoculated cheeses, the main differences were recorded by 7 d of ripening. During the first week, a general increase in the contents of free amino acids was observed in all experimental cheeses except those inoculated with *L. brevis*, which (as happened with the aforementioned two ripening indices) experienced the greatest rise in activity from 7 to 21 d.

In control model cheeses, values obtained for PTASN/TN were generally lower than in their inoculated counterparts, thus indicating that rennet bore little activity on peptides containing a small number of amino acid residues; hence, bacteria present in the inoculated model cheeses are likely to be responsible for generation of nitrogen compounds soluble in 5%(w/w) PTA (Macedo & Malcata, 1997). According to these authors, and seconded by Sousa and Malcata (1997b), a slight decrease in the free amino acid index is likely to be observed during the exponential growth phase of lactic acid bacteria. This conclusion may appear surprising, since one would expect an increase in the total amount of free amino acids released thereby; however, those free amino acids may also be actively consumed for microbial growth, or may alternatively be converted into volatile compounds via their secondary metabolism (Tavaria, Dahl, Carballo, & Malcata, 2002). In the present study, such a trend was not detected, most probably because the nutritional requirements of the strains experimented with our model cheeses did not override generation of the pool of amino acids.

4. Conclusions

The model cheeses manufactured in this study proved suitable in attempts to decouple the individual roles of the proteinases contributed by the rennet and by the native microflora, thus aiding in elucidation of their actual role. The two wild strains assessed were similar in terms of proteolytic patterns in cheese, and they clearly improved release of free amino acids upon incorporation in cheese-making milk. In general, the type of rennet used had no statistically significant impact on secondary proteolysis.

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