Original article

Effect of coagulant type and storage temperature on the functionality of reduced-fat Mozzarella cheese

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Abstract – Reduced-fat low-moisture Mozzarella cheeses (~10%, w/w, fat) were made in triplicate using one of the following coagulants: fermentation-produced chymosin (FPC), *Rhizomucor miehei* proteinase (RMP) or *Rhizomucor pusillus* proteinase (RPP). Coagulants were added to the cheese milk at levels, which gave a curd firmness of 60 Pa in 45 min at 36 °C. Based on their ability to coagulate milk at 54 °C, the heat stability of the enzymes decreased in the following order: RMP > RPP > FPC. Cheese made using each coagulant was stored at 4 or 12 °C. Coagulant type significantly affected the level of primary proteolysis, as measured by levels of pH 4.6 SN, with the RPP giving significantly higher mean levels than the other coagulants at both 4 and 12 °C. However, coagulant type did not significantly affect the firmness of the unheated cheese or the flowability of the cheese on heating at 180 or 280 °C. Increasing storage temperature from 4 °C to 12 °C significantly increased the mean levels of proteolysis and non-expressible serum in the raw cheese and the mean flowability of the heated cheese.

Reduced-fat Mozzarella / coagulant / storage temperature / functionality

Résumé – Effets du type de coagulant et de la température de stockage sur la fonctionnalité de la Mozzarella à faible teneur en lipides. Les fromages Mozzarella à faible humidité et à faible teneur en matière grasse (~10 %, w/w, matière grasse) ont été produits en triple en utilisant un de ces coagulants : chymosine produite par fermentation (FPC), protéinase de *Rhizomucor miehei* (RMP) ou protéinase de *Rhizomucor pusillus* (RPP). Les coagulants ont été ajoutés au lait à une concentration donnée, de manière à obtenir une fermeté de caillé de 60 Pa en 45 min à 36 °C. La stabilité thermique des enzymes, basée sur leur aptitude à coaguler le lait à 54 °C, décroît dans l'ordre suivant : RMP > RPP > FPC. Le fromage produit en utilisant chaque coagulant est stocké à 4 ou à 12 °C. Le type de coagulant affecte de manière significative le niveau de première protéolyse, ceci étant prouvé par les mesures d'azote soluble à pH 4,6, le RPP donne un niveau moyen significativement plus élevé que les autres coagulants à 4 et à 12 °C. Cependant, le type de coagulant n'affecte pas de manière significative la fermeté d'un fromage non chauffé ou la capacité d'écoulement de celui-ci à la cuisson à 180 ou à 280 °C. L'augmentation de la température de stockage de 4 à 12 °C augmente de manière significative le niveau moyen de protéolyse, la quantité de sérum non extractible dans le fromage cru et la capacité d'écoulement moyenne dans le fromage cuit.

Mozzarella allégée en lipides / coagulant / température de stockage / fonctionnalité

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1. INTRODUCTION

Consumer interest in reduced consumption of dietary fat has lead to increased opportunities for reduced-fat cheeses. However, reduction of fat content in Mozzarella cheese impairs the textural and cooking properties of the heated cheese as reflected by a reduction in meltability and flowability [34, 43, 48]. Consequently, various approaches have been employed to accelerate the development of the desired cooking properties (functionality) [12, 13, 31, 42]. These include increases in moisture-in-non-fat substances (MNFS) and free oil, reduction in calcium level, and reduction in intact casein level via elevation of storage temperature [7, 20], the addition of exogenous proteinases from *Bacillus subtilis*, *B. licheniformis* [33], and/ or the use of coagulant of different proteolytic activity [57, 58]. Yun et al. [57] found that the use of *Endothia parasitica* proteinase as coagulant increased proteolysis and heat-induced flowability of low moisture part skim Mozzarella. However, the use of *Endothia parasitica* (now known as *Cryphonectria parasitica*) is generally considered unsuitable for cheese manufacture, as it has been found to reduce cheese yield and cause excessive proteolysis in cheese compared to other coagulants such as chymosin, *Rhizomucor miehei* proteinase, and *Rhizomucor pusillus* proteinase [14, 24]. It is generally agreed that chymosin is largely inactivated during Mozzarella cheese manufacture [8]. The use of coagulants which are more thermostable than chymosin, but less proteolytic than *Cryphonectria parasitica* proteinase, would appear to provide a convenient means of enhancing proteolysis and functionality of reduced-fat Mozzarella. However, to our knowledge, this approach has not been investigated.

This study was undertaken to investigate the combined effects of the use of commercially available coagulants of different thermal lability, and elevated storage temperature, on the functional properties of reduced-fat Mozzarella.

2. MATERIALS AND METHODS

2.1. Coagulant type

Three different coagulants, which according to the suppliers' information had different thermal stabilities, were evaluated. Fermentation produced chymosin (FPC: Chymax plus) and medium thermolabile *Rhizomucor miehei* proteinase (RMP: Hannilase tl 195) were both obtained from Chr. Hansen Ltd. (Little Island, Cork, Ireland). *Rhizomucor pusillus* proteinase (RPP: Emporase), described as being heat stable, was obtained from SKW Biosystems Inc. (Waukesha, WI, USA).

2.2. Standardization of milk clotting activity of different coagulants for cheese manufacture

The milk clotting activity was assessed on a model substrate prepared from extra-low heat skim milk powder (whey protein nitrogen index, 7.5 mg·g^{-1} powder; protein, 34.1% w/w). The powder was produced on a pilot-scale tall-form drier, model TFD-20 (Niro A/S, Copenhagen, Denmark) according to the procedure of Kelly and Kelly [27], using nozzle atomization and inlet and outlet air temperatures of 175–180 °C and 72–74 °C, respectively. The powder was reconstituted at a level of 10% (w/v) in distilled water at 40 °C, using a magnetic hot plate stirrer. Calcium chloride was added to give a final added calcium content of 0.13 mmol·L^{-1} Ca, and NaN$_3$ was added as a preservative at a level of 0.02% (w/w). The reconstituted milk was allowed to hydrate at 20 °C for 20 h. The pH was then adjusted to 6.55 using 0.5 N HCl and the temperature was raised to 31 °C.

Each coagulant was diluted in distilled water (1:20, v/v) and added to the milk at a level within the range recommended by the manufacturers. The rennet coagulation properties were measured using a Formagraph (Model 11700, Foss Electric, Hillerød, Denmark), as described by O'Brien et al. [38] except that the temperature was 31 °C rather than 37 °C. The following parameters were obtained from the displacement/time output signal [1]: (i) rennet coagulation time (RCT) defined as the time at which the time/displacement signal begins to bifurcate at the onset of gelation; and (ii) curd firmness at 30 min, A30, defined as the maximum width of the bifurcation at 30 min after coagulant addition. Using the appropriate

RCT values, the strength of each coagulant was calculated from the RCT and expressed as chymosin units (CU) per mL enzyme, where 1 CU is the enzyme activity required to clot 10 mL substrate in 100 s at 31 °C [14]. The coagulant strength was calculated according to the formula [14]:

$$coagulant\ strength\ (CU \cdot mL^{-1}) =$$

$$\frac{1000 \times 100}{(\mu L\ diluted\ enzyme\ added/dilution\ factor)(RTC \times 60)}$$

where: RCT is the rennet coagulation time in min, and dilution factor is the extent to which the enzyme was diluted with distilled water prior to its addition to the milk. The mean strength $(CU \cdot mL^{-1})$ of each coagulant was thus calculated as 49.6, 44.4 and 165 for the FPC, RMP, and RPP, respectively. The quantities of RPP proteinase and RMP proteinase required to give the same RCT as the FPC, which was the control coagulant and added to the cheese milk at a level of 4.7 mL undiluted enzyme per kg milk protein, were then established (see Sect. 2.4).

2.3. Measuring the thermal sensitivity of the different enzymes

The sensitivity of the different enzymes to temperature was measured by monitoring the changes in their rennet coagulation characteristics (RCT, A30) on changing substrate temperature in the range 28 to 54 °C [6, 9]. The rennet coagulation characteristics were measured on 10% (w/w) reconstituted skim milk powder, as described in Section 2.2. Following overnight holding at 20 °C and pH adjustment, the reconstituted milk was sub-divided into 500 mL portions each of which was tempered to the required temperature by placing in a thermostatically controlled bath at the appropriate temperature for 40 min.

Coagulants were diluted 1 in 20 in distilled water, and added to the milks at the following levels $(\mu L \cdot 10\ mL^{-1})$ to give similar RCT values at 28 °C: FPC, 36; RMP, 40; and RPP, 10. The parameters RCT and A30 were measured using the Formagraph (Model 11700, Foss Electric, Hillerød, Denmark), as described in Section 2.2.

2.4. Manufacture of reduced-fat Mozzarella cheeses

Mid-lactation milk from the Moorepark institute Friesian herd was collected on three separate occasions over a 5-week period. The milk was standardized to a protein-to-fat ratio of 3.4:1 for the manufacture of reduced-fat Mozzarella cheese. The milk was held overnight at <10 °C, pasteurized at 72 °C × 26 s, cooled to 36 °C, and pumped directly to the cheese vats.

Reduced-fat Mozzarella cheeses were manufactured using a dry-salting procedure, as described previously [19]. The milk (~ 450 L) was inoculated with a starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus helveticus* (Chr. Hansens Ltd., Little Island, Cork, Ireland), added at levels of 1.0 and 0.5 $g \cdot 100\ g^{-1}$, respectively. Coagulants were added to the milk on a protein basis, at equivalent levels, which gave a curd firmness of 60 Pa in ~ 40 min at 36 °C for cheese milk containing 3.3% protein, as measured using low-amplitude strain oscillation rheometry [15]. The levels of added coagulants during cheese manufacture, in mL per kg milk protein, were: 4.7, 5.2 and 1.4 for FPC, RMP and RPP. After cutting the gel at a firmness of 60 Pa, the curd/whey mixture was cooked to 38 °C at a rate of 0.25 °C· min^{-1} and pitched at pH 6.15. The curd was cheddared and milled at pH ~ 5.25, dry salted at a level of 41.6 $g \cdot kg^{-1}$, mellowed for 20 min, and plasticized in hot water at 78 °C, as described previously by Guinee et al. [19]. The plasticized curd (58 °C) blocks (~ 22.5 × 10 × 11 cm^3) were cooled to a surface temperature of 24 °C by placing in dilute brine (100 $g \cdot kg^{-1}$, NaCl; pH 5.1; 0.2 $g \cdot kg^{-1}$, $CaCl_2$, 4 °C) for 30 min. The 12 cheeses (2.3 kg) from each vat were vacuum-wrapped and placed at 4 °C. The dry-salting/cooling procedure used in the current study [19] leads to uniform composition throughout the cheese block at 1 d, with no significant differences in salt or moisture contents between parallel layers (22.5 cm length; 1 cm thick) from the surface to the centre of the cheese.

The cheeses made using chymosin, *Rhizomucor miehei*, *Rhizomucor pusillus* proteinase are denoted FPC, RMP and RPP,

respectively. The cheeses stored at 4 and 12 °C, are denoted FPC4, RMP4 and RPP4, and FPC12, RMP12 and RPP12, respectively.

2.5. Sampling of the cheese

Each cheese block was cut into 4 symmetrical quarter portions. One portion was grated to yield particles of <1 mm, in a Krups Rotary 350 food processor fitted with a universal blade. Two of the quarters were cut into 25 mm cubes (Cheese Blocker; Bos Kaasgereedschap, Bodengraven, The Netherlands); some of the resultant cubes were passed through a Hallde RG-350 machine (AB Hallde Maskiner, Kista, Sweden) using the raw food grating disc (K) to yield shreds ≈25 mm long and ≈4 mm diameter. The fourth quarter portion was cut into symmetrical half portions, one of which was cut with a Unika cutter (model WG-300; BOS Kaasgereedschap, Bodengraven, The Netherlands) to give 6.5 mm-thick slices from which discs (45.5 mm) were obtained using a stainless steel borer; the other portion was used for the procurement of cylindrical samples (weight, 15 ± 0.05 g; ~13 mm diam and 33.7 mm height) using a custom-made stainless steel borer.

2.6. Analysis of the uncooked cheese

2.6.1. Composition

Grated cheese (<1 mm particle size) was analyzed for salt, fat, protein, moisture and pH, calcium and phosphorous using standard methods, as described by Guinee et al. [15].

2.6.2. Non-expressible serum per g protein (NESP)

The expressible serum in cheeses stored for 1, 9, 20, 35 and 50 d was determined by centrifugation at 12 500 g at 20 °C, as described by Guinee et al. [21]. The non-expressible serum was then calculated (subtracting the value for expressible serum per 100 g cheese from the moisture content) and is reported as g per g protein [21].

2.6.3. Proteolysis

The levels of primary and secondary proteolysis were monitored by measuring the levels of nitrogen soluble in water at pH 4.6 (pH 4.6SN) and in 5% phosphotungstic acid (50 g·L^{-1}), (PTASN) in grated cheese samples, as described by Guinee et al. [15].

All cheeses were analysed by urea-polyacrylamide gel electrophoresis (PAGE) at 1, 20, 35, and 50 d. PAGE was performed on a Protean II xi vertical slab gel unit (BIO-RAD Laboratories Ltd., Watford, Herts, UK), using a separating and a stacking gel, as described by Feeney et al. [7]. Cheese samples (equivalent to 3.3 mg cheese protein) were dissolved in 1 mL of sample buffer and incubated at 55 °C for ~10 min. The gels (1 mm thick) were pre-run at 280 V for ~40 min prior to sample application and 10 μL samples were loaded using a micro-syringe (Hamilton, Bonaduz, Switzerland). The samples were run through the stacking gel and separating gel at 280 and 300 V, respectively. The gels were stained overnight in Coomassie brilliant blue G250 and de-stained in repeated changes of distilled water.

2.6.4. Cheese rheology

Cheeses were analyzed after 1, 9, 20, 35 and 50 d of storage. For each cheese, a total of 6 cheese cubes (25 mm) were cut from each cheese (Cheese Blocker; Bos Kaasgereedschap, Bodengraven, The Netherlands), placed in an airtight plastic bag and held at 8 °C overnight. The cheese cubes were compressed on a TA-HDi Texture Profile analyser (Stable Micro Systems, Surrey, England) at a rate of 60 mm·min^{-1} at 21 °C. The firmness was defined as the force required to compress to 30% of original height [17].

2.7. Evaluation of cheese functionality on cooking

Cheeses were evaluated after storage for 1, 9, 20, 35 and 50 d. Flowability was measured by (i) a modified Schreiber test [16], defined as the % increase in diameter of a cheese disc on heating at 280 °C for 4 min,

Table I. Composition of reduced-fat Mozzarella cheeses made with different coagulant types.

Composition	Coagulant type			
	FPC	RMP	RPP	SED
Moisture (g·kg⁻¹)	515.6[a]	512.3[a]	513.0[a]	4.12
Fat (g·kg⁻¹)	100.6[a]	101.3[a]	100.1[a]	1.36
Protein (g·kg⁻¹)	332.9[a]	333.3[a]	332.7[a]	2.66
MNFS (g·kg⁻¹)	573.3[a]	570.1[a]	570.1[a]	3.92
FDM (g·kg⁻¹)	207.8[a]	207.8[a]	205.6[a]	1.72
Salt (g·kg⁻¹)	14.7[a]	15.1[a]	15.3[a]	1.03
S/M (g·kg⁻¹)	28.6[a]	29.5[a]	29.9[a]	1.87
Ca (mg·g⁻¹ protein)	251.4[a]	248.7[a]	260.3[a]	8.48
P (mg·g⁻¹ protein)	182.7[a]	180.7[a]	188.4[a]	5.53
pH at 1 d	5.33[a]	5.36[a]	5.36[a]	0.01

FPC: fermentation produced chymosin; RMP: *Rhizomucor miehei* proteinase; RPP: *Rhizomucor pusillus* proteinase; SED: standard error of difference; MNFS: moisture-in-nonfat-substance; FDM: fat-in-dry-matter; S/M: salt-in-moisture. [a] Means within a row sharing a common superscript do not differ significantly.

and (ii) a modified Olson and Price method [40], defined as the % increase in the length of a 15 g cylinder of cheese enclosed in a graduated glass cylindrical tube fitted with a holed rubber bung, containing a 5 mm hole, on heating at 180 °C for 7.5 min.

2.8. Statistical analysis

A randomised complete block design, which incorporated the three treatments (coagulant type: FPC, RMP and RPP) and 3 blocks (replicate trials), was used for analysis of the response variables relating to cheese composition (Tab. I). Analysis of variance (ANOVA) was carried out using a general linear model (GLM) procedure of SAS [44] procedure where the effects of treatment (coagulant type) and replicates were estimated for all response variables. Duncan's multiple comparison test was used as a guide for pair comparisons of the treatment means. The level of significance was determined at $P < 0.05$.

A split-split-plot design was used to determine the effects of coagulant type, storage temperature, storage time and their interactions on the response variables (pH, proteolysis, firmness and flowability) measured at several time points during storage. The main-plot factor was treatment (FPC, RMP and RPP), the subplot factor was storage temperature (4 °C and 12 °C) and the sub-sub-plot factor was storage time. Analysis of variance for the split-split-plot design was carried out using a general linear model (GLM) procedure of SAS [44]. Statistically significant differences ($P < 0.05$) between different treatment levels were determined by Fisher's least significant difference test.

Where flowability of the melted cheese was plotted as a function of pH 4.6 SN, linear regression of data from all cheeses, with intercept, was performed. The significance of the regressions was determined by applying Student's t test to r² with n-2 df where n is the actual number of data points, and df is the degrees of freedom.

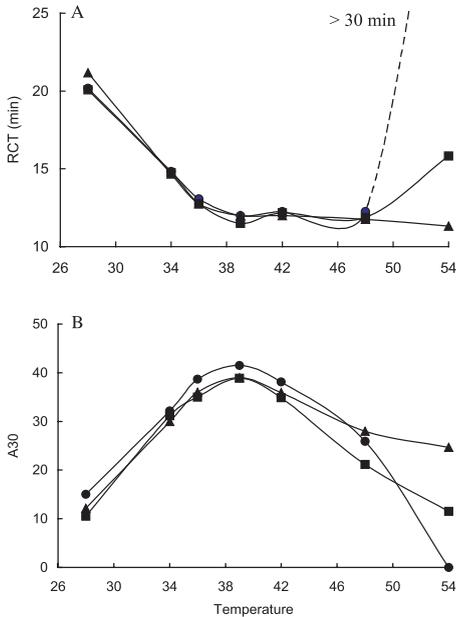The data for PAGE are presented as supportive data but were not statistically analysed.

Figure 1. The effect of temperature on the rennet clotting time (RCT; A) and curd firmness at 30 min (A30; B) of milks to which different coagulants were added: fermentation produced chymosin (FPC: ●), *Rhizomucor miehei* proteinase (RMP: ▲) or *Rhizomucor pusillus* proteinase (RPP: ■). The dashed region (-----) in the RCT curve for the FPC indicates that milk with this enzyme did not coagulate during the 30 min test period at temperatures > 48 °C. Values presented are the means from 4, or more, replicate samples. See text for details of measurements.

3. RESULTS AND DISCUSSION

3.1. Rennet coagulation of milk and thermal stability

The changes in the rennet coagulation parameters, RCT and A30, for the different coagulants as a function of substrate temperature are shown in Figure 1. The rennet coagulability of all enzymes was enhanced by raising the substrate temperature from 28 to 42 °C, as reflected by the decreases in RCT and increases in A30. The thermal stability of the enzymes decreased in the fol-

lowing order: RMP > RPP > FPC. Hence, the FPC failed to coagulate milk at temperatures ≥ 54 °C. In contrast, the RMP remained quite active at 54 °C, with RCT and A30 values comparable to those at 32 °C.

3.2. Cheese composition

The mean compositions of the FPC, RMP or RPP cheeses are given in Table I and are typical for reduced fat Mozzarella cheeses [43, 45, 48]. Similar to the findings of Dave et al. [3] and Oberg et al. [37], the coagulant type did not significantly effect cheese composition. This is contrary to the findings of Tunick et al. [49] who reported a significantly lower MNFS content in low fat Mozzarella cheese manufactured with FPC than in the cheeses made with RMP, *C. parasitica,* or calf rennet. Yun et al. [57] also reported numerically small, but statistically significant, differences in the levels of salt and FDM between Mozzarella cheeses made with *Endothia parasitica*, RMP or FPC.

3.3. Age related changes in pH

There was no effect of coagulant type on pH at day 1 (Tab. I) or on age related changes in pH during storage (Tab. II, Fig. 2). However, there was a significant effect of storage temperature ($P < 0.001$) and of time ($P = 0.001$) on the pH of the reduced fat cheeses (Fig. 2, Tab. II). None of the interactions were significant (Tab. II). Increasing storage temperature from 4 to 12 °C resulted in a decrease in mean pH. The decrease in pH with storage temperature may reflect an increase in precipitation of colloidal calcium phosphate, the solubility of which decreases with increasing temperature [52, 54]. An increase in calcium phosphate precipitation would be conducive to a liberation of H^+ ions from the phosphate anions and a concomitant increase in hydrogen ion activity. A change in the buffering capacity with storage temperature may also contribute to the differences in pH between cheeses stored at 4 and 12 °C [30]. The pH of all cheeses increased during storage which concurs with the age related changes in pH observed by Metzger et al. [34] in

Table II. Degrees of freedom (df), mean squares (MS) and probabilities (*P*) for aggregated changes in pH, non-expressible serum per gram protein (NESP), proteolysis as measured by pH 4.6-soluble N (pH 4.6SN) and 5% phosphotungstic acid-soluble N (PTASN), and firmness in reduced-fat Mozzarella cheese made using different coagulants and stored at 4 or 12 °C.

| Factors	pH			NESP			pH 4.6SN			PTASN			Firmness		
	df	MS	*P*	df	MS	*P*	df	MS	*P*	df	MS	*P*	df	MS	*P*
Main Plot															
Coagulant type	2	0.0071	0.5788	2	0.0014	0.7680	2	19.1553	0.0188	2	0.0357	0.429	2	14498.9	0.2884
Error	4	0.0113		4	0.0050		4	1.5223		4	0.0339		4	8407.6	
Split Plot															
Temperature interaction	1	0.0859	0.0009	1	0.0001	0.0073	1	93.9039	<0.0001	1	2.0627	<0.0001	1	116637	0.0004
Temperature × Coagulant	2	0.0018	0.4999	2	0.000009	0.3215	2	1.8140	0.0994	2	0.0254	0.2998	2	1403.1	0.5818
Error	6	0.0023		6	0.000007		6	0.5219		6	0.0171		6	2363.7	
Split-Split Plot															
Time interaction	4	0.0213	0.0013	4	0.1641	<0.0001	4	117.241	<0.0001	4	1.5700	<0.0001	4	126264	<0.0001
Coagulant × Time	8	0.0002	0.9999	8	0.000150	0.9509	8	2.8680	0.0119	8	0.0111	0.3372	8	2524.1	0.4119
Temperature × Time	4	0.0087	0.0888	4	0.000100	0.9193	4	20.0574	<0.0001	4	0.5533	<0.0001	4	16709.7	0.0002
Coagulant × Temp × Time	8	0.0007	0.9945	8	0.000009	1.0000	8	0.5114	0.7845	8	0.0070	0.6354	8	1168.5	0.8585
Error	48	0.0040		48	0.000400		24	0.8825		24	0.0092		45	2395.9	
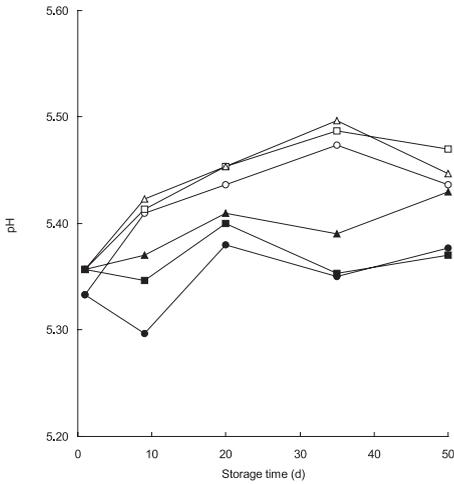
Figure 2. Age-related changes in pH of reduced-fat Mozzarella cheeses made with fermentation produced chymosin (FPC: ○,●), *Rhizomucor miehei* proteinase (RMP: □,■) or *Rhizomucor pusillus* proteinase (RPP: △,▲) and stored at 4 (○,□,△) or 12 °C (●,■,▲). Values presented are the means from 3 replicate trials.

Figure 3. Age-related changes in non-expressible serum expressed per g protein (NESP) in reduced-fat Mozzarella cheeses made with fermentation produced chymosin (FPC:○,●), *Rhizomucor miehei* proteinase (RMP: □,■) or *Rhizomucor pusillus* proteinase (RPP:△,▲) and stored at 4 (○,□,△) or 12 °C (●,■,▲). Values presented are the means from 3 replicate trials.

reduced-fat Mozzarella and Guinee et al. [21] and Guo et al. [23] in low-moisture part-skim Mozzarella.

3.4. Non-expressible serum (NESP)

The level of non-expressible serum, as expressed as g·g^{-1} protein (NESP), is an indicator of water holding capacity (WHC) of cheese with a high level indicating a high water holding capacity [21, 28]. The mean level of NESP in cheeses stored at 12 °C was significantly higher than that in cheese stored at 4 °C ($P < 0.01$) (Tab. II). This trend concurs with that of Guinee et al. [20] who reported a progressive decrease in expressible serum as the storage temperature was increased from 0 to 15 °C. As observed in previous studies for reduced-fat Mozzarella [34, 45], there was a significant increase in the mean level of NESP during storage (Fig. 3, Tab. II) with the greatest increase occurring at < 9 d, and little, or no, change after 20 d. Coagulant type did not significantly affect the level of NESP nor were there any significant interactions. This
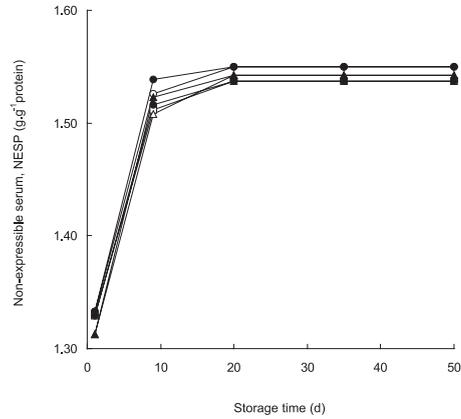
trend is somewhat surprising as storage temperature and coagulant type, as discussed below, affected the level of proteolysis. The level of NESP generally increases with proteolysis, as reflected by the increase in NESP with maturation time or levels of pH 4.6SN [20, 22]. However, to our knowledge, there is little, or no, published information on the effect of proteolysis per se on the level of NESP in cheese. Moreover, the level of NESP in cheese is also affected by other parameters such as level of NaCl and ratio of soluble-to-colloidal calcium [22, 23].

3.5. Proteolysis

3.5.1. Urea-PAGE

The Urea-PAGE gel electrophoretogram of the cheeses from trial 2 is shown in Figure 4 and is typical of the cheeses from trials 1 and 3. Storage at both temperatures resulted in a progressive degradation of α_{s1}- and β-caseins with the extent of hydrolysis of α_{s1}-casein being greater than that of the
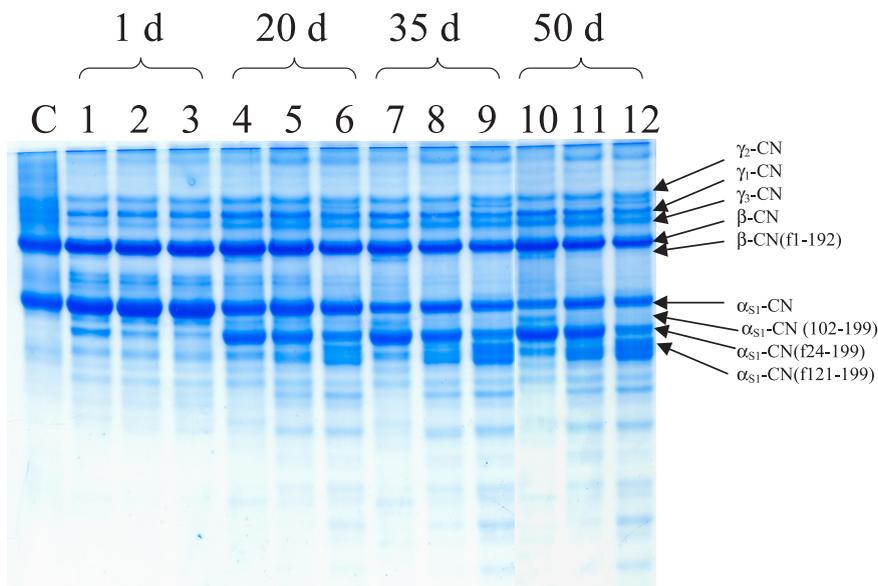
Figure 4. Urea-PAGE analysis of sodium caseinate (C) and reduced-fat Mozzarella cheeses made using fermentation produced chymosin (FPC, lanes 1, 4, 7 and 10), *Rhizomucor miehei* proteinase (RMP, lanes 2, 5, 8 and 11) or *Rhizomucor pusillus* proteinase (RPP, lanes 3, 6, 9 and 12) and stored at 12 °C for 1, 20, 35 and 50 d.

latter (Fig. 4). The hydrolysis products α_{s1}-casein (f 124-199), β-casein (f 1-192) and γ-caseins accumulated during storage to an extent dependent on the coagulant used and storage temperature. These degradation patterns are consistent with those of previous studies for low-moisture part-skim Mozzarella made using fermentation produced chymosin [7, 8, 45, 57].

At all storage times, the levels of intact α_{s1}- and β-caseins casein were similar in cheeses made with the different coagulants. However, the level of age-related degradation of the primary α_{s1}-casein hydrolysis product, α_{s1}-casein (f24-199), was highest for the RPP cheese and lowest for the FPC cheese. The greater degradation of α_{s1}-casein (f24-199) in the cheeses containing the microbial coagulants coincided with high levels of peptides with higher electrophoretic mobility in the RPP cheese, and to lesser extent, in the RMP cheese. Similar to Feeney et al. [7], the level of α_{s1}-casein (f102-199) was present at very low concentrations in all cheeses, with the level

decreasing with storage time especially in the RPP and RMP cheeses stored at 12 °C. The varying degrees of hydrolysis of α_{s1}-casein (f24-199) probably reflects the combined effects of differences between the enzymes in relation to their intrinsic proteolytic activities [26, 41, 46, 51, 57], the effect of the cheese environment on their proteolytic activities [10, 35, 36], and the thermolability of their proteolytic activities during the curd plastization process [2, 25, 47, 55].

In agreement with previous studies on Mozzarella made using starter culture [7, 45, 57], there was comparatively little degradation of β-casein in all cheeses. However, the hydrolysis products, γ-caseins, were evident, in all cheeses. The level of γ_1-casein was lower in the RPP cheese than that in the FPC and RMP cheeses at storage times $\geq$ 20 d.

In agreement with previous studies [7], increasing the storage temperature from 4 to 12 °C significantly increased the degree of degradation of α_{s1}-casein at storage times $\geq$ 1 d, and only slightly increased the

hydrolysis of β-casein (data not shown). However, temperature did not influence the profile of electrophoretic products.

3.5.2. Changes in pH 4.6SN and PTASN

The mean levels of pH 4.6SN and PTASN increased significantly in all cheeses during storage (Fig. 5, Tab. II). The levels of pH 4.6SN in the FPC4 cheeses were typical in magnitude to those previously reported for reduced-fat (~10%, w/w) cultured Mozzarella stored at 4 °C for similar times [45], and lower than the levels reported for low-moisture part-skim (~20% fat) Mozzarella [18, 57].

The mean levels of pH 4.6SN were significantly affected by coagulant type, storage temperature, and the interactions, both, between coagulant type and time, and between storage temperature and time (Tab. II). At times ≥ 20 d, the development of pH 4.6SN were markedly influenced by coagulant, with the levels in the in the RPP cheeses being significantly higher than those in the FPC and RMP cheeses, which were similar. Significant effects of the interaction between coagulant type and storage time on primary proteolysis in Mozzarella cheese were also observed by Yun et al. [57] and Dave et al. [3].

Various studies have found that the heat stabilities of coagulants decreases in the following order: RMP > RPP > veal rennet/chymosin [25, 47] on heating at pH 5.2. However, the thermostability of the coagulants (rennets) is affected by many parameters including the degree of modification (e.g. by oxidation of methionine residues in the molecule) [2], pH, temperature and time [11, 47]. The current results on the effect of substrate temperature on the rennet coagulability of milk (Fig. 1) suggest that the *Rhizomucor pusillus* proteinase used in the current study had a lower thermal stability than that of the *Rhizomucor miehei* proteinase, probably as a consequence of different degrees of modification. Hence, the significantly higher levels of pH 4.6SN for the RPP cheese compared to RMP cheese suggests that that the RPP has a higher ratio of proteolytic activity-to-milk clotting activ-
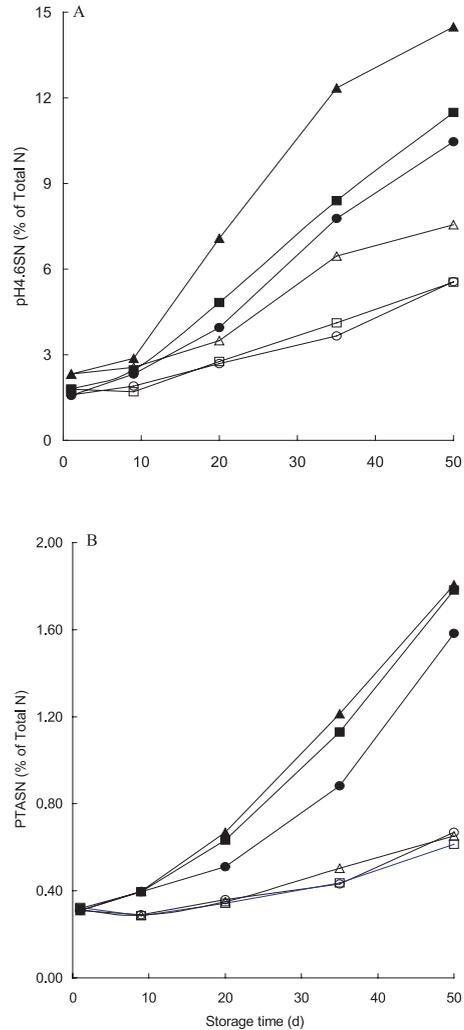

Figure 5. Age-related changes in the concentration of pH 4.6SN (A) and 5% PTASN (B) in reduced-fat Mozzarella cheeses made with fermentation produced chymosin (FPC:○,●), *Rhizomucor miehei* proteinase (RMP: □,■) or *Rhizomucor pusillus* proteinase (RPP:△,▲) and stored at 4 (○,□,△) or 12 °C (●,■,▲). Values presented are the means from 3 replicate trials.

ity than the RMP. The trend in proteolysis is consistent with that generally reported in the literature, which despite inter-study discrepancies, show that *Rhizomucor pusillus*

proteinase tends to give higher levels of proteolysis in cheese than *Rhizomucor miehei* proteinase or chymosin [14]. The absence of significant differences between the RMP and FPC cheeses concurs with the findings of Yun et al. [57] which showed no significant differences in the levels of pH 4.6SN levels in low moisture Mozzarella cheeses made using fermentation produced chymosin or *Rhizomucor miehei* proteinase.

In agreement with the trends noted for PAGE, the mean levels of pH 4.6SN increased significantly on raising the storage temperature from 4 to 12 °C at storage times ≥ 20 d. This trend concurs with that reported for Mozzarella [7] and other cheeses [56].

Similar to the results of Yun et al. [57], coagulant type did not significantly influence the level of secondary proteolysis, as measured by formation of PTASN. This trend is expected as residual coagulant in cheese mainly contributes to primary proteolysis, rather than to secondary proteolysis [39, 53]. There was a significant effect of the interaction between storage temperature and time on PTASN levels. The levels of PTASN in the cheeses stored at 12 °C were significantly higher than those in the cheeses stored at 4 °C at times >1 d. Similar effects of temperature on the formation of PTASN were reported by Feeney et al. [7].

3.6. Rheology

Storage temperature, time and their interaction significantly affected the firmness of the cheese. The mean firmness of the cheeses stored at 12 °C decreased significantly during storage; that of cheeses stored at 4 °C decreased but non significantly. This decrease in mean firmness during storage for 50 d at 12 °C is comparable to that observed by Guinee et al. [20] on raising the storage temperature for Mozzarella from 4 to 10 °C. The decrease in firmness with temperature is consistent with the greater degree of proteolysis and lower degree of intact (unhydrolysed) casein [4, 5, 17]. The small age-related decrease in firmness at 4 °C is typical for reduced-fat Mozzarella [34, 45, 50], and probably reflects the relatively low level of proteolysis (Fig. 5).
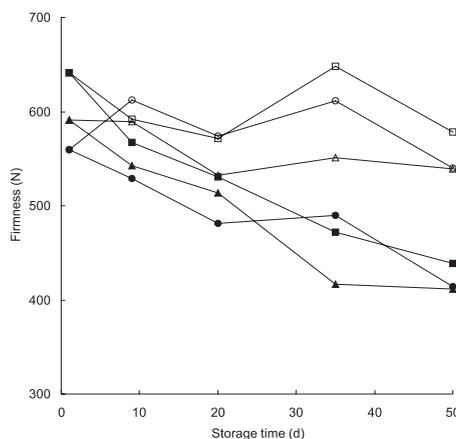

Figure 6. Age-related changes in the firmness of reduced-fat Mozzarella cheeses made with fermentation produced chymosin (FPC: ○,●), *Rhizomucor miehei* proteinase (RMP: □,■) or *Rhizomucor pusillus* proteinase (RPP: △,▲) and stored at 4 (○,□,△) or 12 °C (●,■,▲). Values presented are the means from 3 replicate trials.

Coagulant type did not significantly affect the firmness of the cheese (Fig. 6, Tab. II). Similarly, Yun et al. [58] reported no significant difference in the firmness of Mozzarella cheeses made with different coagulants, despite the relatively large differences in primary proteolysis between cheese made using *Endothia parasitica* proteinase and FPC or RMP.

3.7. Functionality on cooking

There was a significant effect of storage time, temperature and their interaction on the storage-related changes in flowability (Fig. 7, Tab. III). The flowability of all cheeses increased during maturation, a trend consistent with the increases in non-expressible serum and primary proteolysis [22, 23, 32, 45]. At times ≥ 20 d, the mean flowability of cheeses stored at 12 °C was significantly higher than that of cheese stored at 4 °C. Similar trends with storage temperature were reported by Guinee et al. [20] and were attributed to a decrease in the level of intact casein at the elevated storage temperatures. Thus, there was a significant

Table III. Degrees of freedom (df), mean squares (MS) and probabilities (*P*) for aggregated changes in flow, as measured by both modified Olson and Price [40], and Schreiber methods [16], of heated reduced-fat Mozzarella cheese made using different coagulants and stored at 4 or 12 °C.

Factors	Flow: modified Olson and Price			Flow: modified Schreiber		
	df	MS	*P*	df	MS	*P*
Main Plot						
Coagulant type	2	2630.169	0.1891	2	28.591	0.5166
Error	4	1011.672		4	36.535	
Split Plot						
Temperature interaction	1	41744.162	<0.0001	1	762.157	0.0004
Temperature × Coagulant	2	236.386	0.0795	2	65.175	0.0686
Error	6	59.452		6	15.053	
Split-Split Plot						
Time interaction	4	104194.571	<0.0001	4	2603.553	<0.0001
Coagulant × Time	8	495.556	0.8633	8	45.972	0.4808
Temperature × Time	4	5046.037	0.002	4	239.613	0.0020
Coagulant × Temp × Time	8	329.892	0.9543	8	29.394	0.7629
Error	48	1029.395		45	48.025	

relationship between the levels of pH 4.6SN and flowability, using both the modified Schreiber (r = 0.83, df = 64) and modified Olson and Price (r = 0.86, df = 64) methods (Fig. 8).

In agreement with the findings of Yun et al. [58], coagulant type did not significantly affect flowability. However, the RPP cheeses had numerically higher mean levels of flowability than the RMP and FPC cheeses, as measured by both methods. These results suggest that the increases in primary proteolysis on using the RPP coagulant compared to the RMP and FPC (~ 2 and 4.5% pH 4.6SN/total N at 4 and 12 °C; Fig. 5A) were not sufficiently large enough to induce a significant increase in heat-induced flowability. In contrast to the current results, Yun et al. [58], found a significant interaction between coagulant type and storage time for Mozzarella cheeses made using fermentation produced chymosin, *Rhizomucor miehei* proteinase, or *Endothia parasitica* proteinase. However,

the difference in the levels of pH 4.6SN between cheeses made with *Endothia parasitica* proteinase and the other coagulants on storing at 4 °C in the latter study was markedly larger than that between RPP and the other coagulants in the current study, i.e., 8% versus 2% pH 4.6SN/total N. Moreover, Yun et al. [57] reported differences in the type of proteolysis between coagulants, with *Endothia parasitica* proteinase giving markedly more extensive degradation of β-CN than the other two coagulants; such differences in the type of proteolysis was not observed in the current study.

There was a significant effect of time, storage temperature and their interaction on flowability (Fig. 7, Tab. III). The mean flowabilities as measured by both the modified Schreiber and Olson and Price methods were significantly higher in cheeses stored at 12 °C than those at 4 °C at d ≥ 20. This trend was expected as a parallel interaction between storage temperature and time was evident for the level of pH 4.6SN at d ≥ 20.
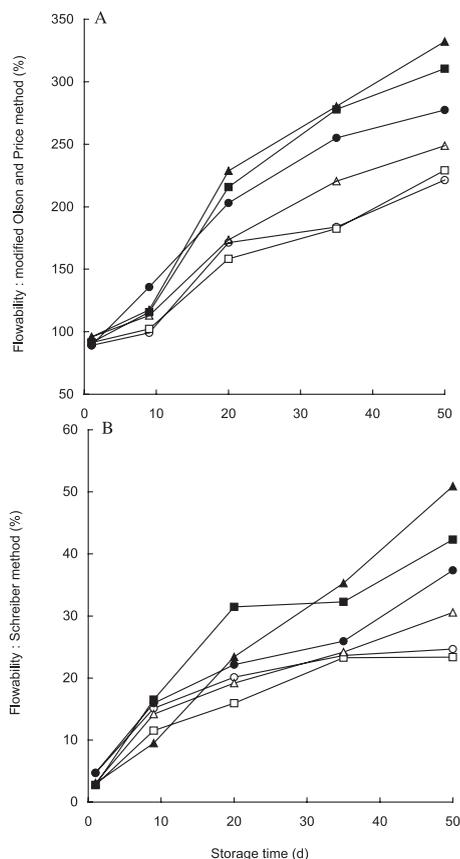
Figure 7. Age-related changes in flowability, as measured using the modified Olson and Price method (A) or the modified Schreiber method (B) of reduced-fat Mozzarella cheeses made with fermentation produced chymosin (FPC: ○,●), *Rhizomucor miehei* proteinase (RMP: □,■) or *Rhizomucor pusillus* proteinase (RPP:△,▲) and stored at 4 (○,□,△) or 12 °C (●,■,▲). Values presented are the means from 3 replicate trials.

Figure 8. Relationship between the concentration of pH 4.6SN and flowability as measured by (A) the modified Schreiber method and (B) the modified Olson and Price method. The data were obtained from reduced-fat Mozzarella cheeses manufactured in three replicate trials with fermentation produced chymosin, *Rhizomucor miehei* proteinase or *Rhizomucor pusillus* proteinase and stored at 4 or 12 °C for up to 50 d; regression lines with intercept shown. Regression was significant ($P < 0.05$) in all cases.

Similar trends were observed by Guinee et al. [20], who noted that the higher flowability in Mozzarella cheese stored at the higher storage temperatures (e.g. 4 compared to 10 or 15 °C) were associated with higher levels of proteolysis (Feeney et al. [7]) and lower levels of intact casein. Moreover, the higher mean levels of NESP, and thus the higher water binding capacity, 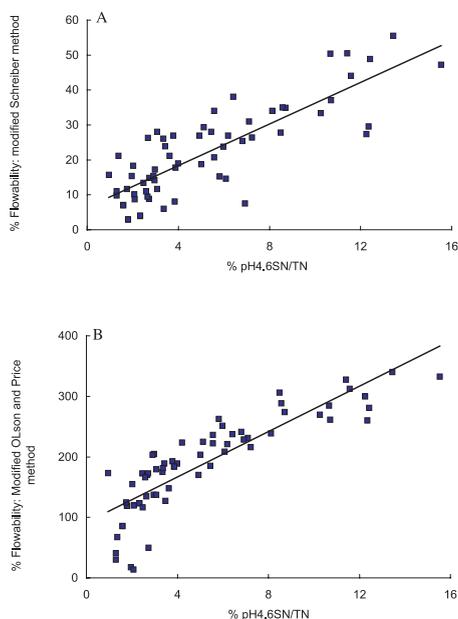in the cheese stored at 12 °C would be more conducive to higher flowability than in the cheeses stored at 4 °C [28, 29, 32]. Heat-induced flow, or spread, may be defined as heat-induced displacement of adjoining layers of the casein matrix, as facilitated by liquefaction and coalescence of fat, which lubricates the surfaces of protein matrix, and the desired ratio of viscous-to-elastic character of the matrix [12, 13]. Increasing the levels of NESP and proteolyis would lead to a more-fluid and less-elastic matrix and, hence, a greater degree of heat-induced flow.

4. CONCLUSIONS

Rennet coagulation studies on reconstituted skim powder using the Formagraph

method indicated differences in the thermal stability between commercial coagulants; based on the RCT and A30 coagulation parameters, the thermal stability decreased in the following order: RMP > RPP > FPC. When used for the manufacture of reduced-fat Mozzarella, coagulant type significantly affected the level of primary proteolysis, but otherwise had little impact on the composition of the cheese. For cheeses stored at both 4 and 12 °C, the use of RPP resulted in significantly higher mean levels of primary proteolysis, as measured by levels of pH 4.6SN, than the other coagulants. However, the magnitude of the increase (~ 2–5% total N, depending on storage temperature) was not sufficiently large enough to affect a significant increase in the firmness of the unheated cheese or the flowability of the cheese on heating at 180 or 280 °C. In agreement with previous results [8, 21], the elevation of storage temperature proved to be an effective means of improving the quality of the cheese, by reducing the firmness and increasing the heat-induced flowability. However, there was no significant synergistic effect between elevated storage temperature and coagulant type on the quality of the cheese.

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