# Influence of chymosin type and curd scalding temperature on proteolysis of hard cooked cheeses

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**Shortened title: Residual coagulant and proteolysis of hard cheeses**

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**Summary**

In this work, we studied the influence of the type of coagulant enzyme and the curd scalding temperature on the proteolysis and residual coagulant and plasmin activities of a cooked cheese, Reggianito, in the interest of reducing ripening time. A two-factor experimental design was applied in two levels: type of coagulant enzyme, bovine chymosin or camel chymosin, and curd scalding temperature, 50 or 56 °C. The experimental treatments were applied in Reggianito cheese making experiments, and the samples were ripened for 90 d at 12 ºC. Scalding temperature influenced residual coagulant activity; the cheeses cooked at 50 °C had significantly higher activity than those treated at 56 °C. In contrast, scalding temperature did not modify plasmin activity. Proteolysis was primarily affected by curd cooking temperature because chymosin-mediated hydrolysis of $\alpha_{s1}$ casein was slower in cheeses treated at 56 °C. Additionally, the nitrogen content in the cheese soluble fractions was consistently lower in the cheeses scalded at 56 °C than those cooked at 50 °C. A significant influence from the type of coagulant enzyme was observed, especially in the nitrogen fractions and peptide profiles, which demonstrated that camel chymosin was slightly less proteolytic; however, these differences were lower than those caused by the scalding temperature.

**Keywords:** camel chymosin, bovine chymosin, residual coagulant activity, scalding temperature, proteolysis

Hard cooked cheeses, such as Reggianito and Sardo, derived from Italian Grana and Parmesan cheeses, have existed in Argentina since the late 18$^{th}$ century. The cheeses were introduced by Italian immigrants and have since become different from the original varieties but also different from Parmesan-like American or generic products (Zannoni et al. 1994). In hard cooked cheeses, ripening is a long process that takes from 6 to 24 months, and involves extensive proteolysis and moderate lipolysis (Candioti et al. 2002; Fox, 2003; Gobetti & DiCagno, 2003). During such a long ripening time, the available carbohydrates may also undergo changes, and the catabolism of free amino acids and fatty acids takes place (McSweeney & Sousa, 2000; Tavaria et al. 2002). These transformations influence cheese texture and functionality, and they are responsible for the characteristic flavour development (McSweeney, 2004; Sgarbi et al., 2013)

Several strategies have been proposed to accelerate Reggianito cheese ripening. These strategies include ripening at increased temperatures (Sihufe et al. 2010; Ceruti et al. 2012), and while the results are encouraging, this approach has not been popular with industry decision-makers so far. Another approach involves the addition of exogenous enzymes, such as proteases, carboxypeptidases and lipases (El Soda & Awad, 2003; Wilkinson & Kilcawley, 2005; Azarnia et al. 2011). To date, mostly lipases have been successfully applied in the cheese industry for the accelerated ripening of hard cooked cheese. In general, exogenous proteases are not suitable, as they can cause shifts in the standard patterns of proteolysis and consequently result in defective texture or flavour (Wilkinson & Kilcawley, 2005). Furthermore, the addition of exogenous enzymes in cheese milk is an expensive technology, as enzymes are typically high-cost ingredients and most are lost in the whey in high proportions (Upadhyay & McSweeney, 2003; Wilkinson & Kilcawley, 2005).

74 Therefore, we took a different approach for the acceleration of Reggianito ripening that
75 consisted of increasing the activity of the proteolytic and lipolytic enzymes that are
76 usually present in the cheese, by using either indigenous or frequently added enzymes.
77 Proteolytic enzymes, such as the coagulant added to milk during cheese making, and
78 plasmin, a native milk protease, are normally active in cheeses and contribute to casein
79 degradation, depending on the cheese type (Bansal et al. 2007; Ismail & Nielsen, 2010)
80 The residual coagulant enzyme is one of the proteolytic agents that contribute to
81 proteolysis during the ripening of most cheeses, particularly in varieties with low or
82 medium scalding temperatures (Fox, 2003). Most of the coagulant is lost in the whey
83 during the draining process. However, the proportion of enzyme retained in the curd is
84 dependent on enzyme type, draining pH, scalding temperature and curd moisture (Sousa
85 et al. 2001; McSweeney, 2004; Upadhyay et al. 2004). During cheese ripening, the
86 coagulant hydrolyses mostly $\alpha_{s1}$-casein at the $Phe_{23}$-$Phe_{24}$ bond to give peptides $\alpha_{s1}$ (f1-
87 23) and $\alpha_{s1}$ (f24-199) or $\alpha_{s1}$-I (Carles & Ribadeau-Dumas, 1985; McSweeney, 2004). The
88 contribution of residual coagulant to proteolysis during cheese ripening has been
89 elucidated for the major varieties of cheeses, but it is less known for the hard cooked
90 cheeses. For this cheese type, the presence of peptide $\alpha_{s1}$-I has been attributed either to
91 chymosin hydrolysis in the vat before curd scalding (Chianese et al. 1997; Gaiaschi et al.
92 2000), to late coagulant action during ripening that it is dependent on the curd scalding
93 temperature (Delacroix Buchet & Fournier, 1992; Kindstedt et al. 1995), or to coagulant
94 enzyme reactivation (Hynes et al. 2004). Other authors claim that proteolysis caused by
95 starter cultures of *Lactobacillus helveticus* should not be discarded as the source of $\alpha_{s1}$-I
96 in Swiss-type cheeses (Sadat-Mekmene et al. 2013).
97 In Argentina, Reggianito cheese was initially made with calf rennet, which was produced
98 locally and was most likely very different from the European standards. During the $20^{th}$

century, adult bovine coagulant was typically applied for this cheese type because porcine pepsin and other substitutes of calf rennet were never widespread in Argentina. Finally, in the 1990s, bovine chymosin produced by fermentation became the standard for Reggianito cheese for large to mid-size dairy industries, as it is the industrial gold standard for cheese manufacturing (Yegin & Dekker, 2013). Recently, Kappeler et al. (2006) expressed the gene for camel (*Camelus dromedarius*) chymosin in *Aspergillus niger* and produced camel chymosin by fermentation. Fermentation-produced camel chymosin has been extensively marketed in all cheese producing countries, including Argentina, because of its higher clotting activity and lower general proteolytic activity compared to bovine chymosin (Kappeler et al. 2006; Jensen et al. 2013). While in Cheddar and mozzarella cheeses, the replacement of bovine chymosin by camel chymosin has been satisfactory (Bansal et al. 2009a; Govindasamy-Lucey et al. 2010; Moynihan et al. 2014), the introduction of this coagulant for Reggianito cheese in Argentina has occurred without prior research.

In this paper, we studied the influence of the curd scalding temperature on chymosin and plasmin activity and their related proteolysis during the ripening of Reggianito cheese for the investigation of ripening acceleration. For this purpose, we assayed two chymosin types that are most commonly applied for Reggianito cheese in the Argentinean dairy industry: bovine and camel chymosins produced by fermentation.

## Materials and Methods

### *Experimental Design*

The influence of two different factors on cheese proteolysis was assessed across the time of ripening: coagulant type and curd scalding temperature. Both factors were studied at

two levels, which gave a total of 4 experimental treatments. Recombinant bovine (**CH**) and camel (**CA**) chymosins were tested (Chy-Max ultra, 950 IMCU and Chy Max M, 740 IMCU, respectively), both provided by Chr. Hansen Argentina (Quilmes, Argentina). Two different curd scalding temperatures were applied: 50 and 56 ºC. These target temperatures were chosen as follows: technical literature and interviews with cheese-makers accounts for Reggianito cooking temperature most frequently applied is 52-54 ºC; however the rank is wider, as Argentinean cheeses are not as standardized as their Italian antecedents. In this sense, 56 ºC is the highest temperature registered for Reggianito cooking temperature, and 50 ºC is lower than usual, but proposed here as an approach to increase residual activity of coagulant and accelerate proteolysis. The cheeses were identified as CA-50ºC, CA-56ºC, CH-50ºC or CH-56ºC. Each cheese experiment trial included the four treatments and was carried out the same day in parallel vats, using the same batch of milk. Four replicates of the cheese makings were performed on four different days with different milk.

***Cheese Making***

The Reggianito Argentino cheeses were manufactured according to the standard process (Gallino, 1994; Meinardi et al. 2002) adapted to laboratory-scale vats to obtain miniature hard-cooked cheeses. This model cheese has been validated and applied previously (Hynes et al. 2004; Vélez et al. 2010; Vélez, 2013). An ensemble of four vats, equipped with a system for simultaneous heating and cooling, was used for the cheese making. Raw bulk milk, pH 6.65 ± 0.05 and 18 ± 1 ºD acidity, was supplied by a nearby dairy plant (Milkaut SA, Santa Fe, Argentina). The milk was standardized to 2.5 g/100 ml fat and batch-pasteurized at 65 ºC for 20 min. After cooling to 33 ºC, $CaCl_2$ was added to a final concentration of 0.2 g/l. The milk pH was adjusted with lactic acid (1.5 g/100 ml) to the

target value for Reggianito cheeses (6.30). Then, a mixed commercial starter of *Lactobacillus helveticus* (Chr. Hansen Argentina, Quilmes, Argentina) was added at a concentration of $10^6$ cfu/ml of milk. Direct acidification and the direct-to-vat thermophilic lactobacilli starter is a methodology that replaces the traditional "natural" whey culture, which is still used by medium and small dairy plants for Reggianito cheese making (Candioti et al. 2002). After manual stirring, each coagulant was added, in an adequate dose to obtain equivalent milk-clotting times after being dissolved in 10 ml of distilled water. After 18 to 20 min, the coagulum attained the appropriate firmness and was cut to adequate grain size (approximately half a rice grain). The mixture of curd particles and whey was gently stirred and heated at 0.5 °C/min until it reached 44 °C to reduce moisture in the curd grains ("curd drying" step). To obtain similar moisture content in all samples, this step was more prolonged for the cheeses cooked at 50 °C. The mixture was then more rapidly heated to 50 or 56 °C (1 °C/min). The curds were maintained at the target temperature for three minutes (cooking step). After cooking, the curds were separated from the whey and placed in molds, pressed for 20 h, and brined for 12 h in saturated brine at 12 °C. The cheeses were ripened for 90 days at 85% relative humidity and 12 °C, but after the first 2 weeks they were vacuum packed in plastic film to avoid over drying. This time was selecting according to previous experiments for the validation of the model (Vélez et al. 2013) Ripening time was fixed in 90 days taking into account gross composition evolution and biochemistry of the ripening (Hynes et al. 2004; Vélez et al. 2010; Vélez, 2013; Vélez et al. 2015, sent). Cylindrical samples of the cheeses were taken on days 6, 50 and 90. The superficial section was discarded (1 cm) and the rest of the cheese cylinder was grated and mixed (IDF, 1995), and these samples were stored at −18 °C until analysis.

174 ***Gross Composition and pH of Cheeses***

175 Gross composition was checked in one single point (50 d of ripening) as the purpose of
176 the assay was to monitor that all the cheeses were comparable and appropriate for
177 modelling hard cooked cheeses. Total protein, fat matter and dry matter were analysed
178 according to IDF standards (IDF, 1993; IDF, 1997; IDF, 1982). As for pH, it was recorded
179 along ripening time as described by Bradley et al. (1993) at 6, 50 and 90 d old cheeses.
180 All analyses were performed in duplicate.

181

182 ***Residual Coagulant and Plasmin activity***

183 The residual coagulant activity in the cheese samples was analysed according to the
184 method of Hurley et al. (1999), with some modifications, at 6, 50 and 90 d of ripening.
185 This method quantifies the hydrolysis of a synthetic chromophoric heptapeptide substrate
186 (Pro-Thr-Glu-Phe-[NO2-Phe]-Arg-Leu) (Bachem California, Inc., Torrance, USA).
187 From this substrate, aspartic proteases are able to release the tripeptide ([NO2-Phe]-Arg-
188 Leu) in proportion with their activity; this tripeptide is then quantified by RP-HPLC.
189 Finely grated cheese samples (250 mg each) were dispersed in 5 ml of water and
190 incubated at 37 ºC for 60 min with periodical stirring in a vortex. Then, the samples were
191 centrifuged at 1000 g for 3 min, and the supernatant (70 µl) was seeded into a reaction
192 mixture consisting of 30 µl of a mother solution of the heptapeptide (1 mg/ml) diluted
193 into 200 µl of 0.1 M formate buffer, pH 4.1. After incubation at 37 ºC for 6 h, the
194 enzymatic reaction was stopped by heating at 70 ºC for 10 min. A volume of 60 µl that
195 was previously filtered through 0.45 µm pore diameter membrane (Millex, Millipore, São
196 Paulo, Brazil) was injected into the HPLC. The HPLC system consisted of a quaternary
197 pump, an online degasser, and a UV/visible detector (all Series 200, Perkin Elmer,
198 Norwalk, CT). An interface module connected to a computer was used for the acquisition

of chromatographic data with the Turbochrom software (Perkin Elmer). The analysis was performed on an Aquapore OD-300 (C18, 5 nm−300 Å, 250 mm × 4.6 mm) analytical column (Perkin Elmer) using a gradient between two mobile phases: A ($H_2O$-trifluoroacetic acid (TFA; 1000:1, vol/vol) and B (acetonitrile-$H_2O$-TFA, 600:400:1, vol/vol/vol). The column was initially equilibrated with 15% B and maintained in this condition for 5 min after injection of the sample. Then, a linear gradient was utilized to increase from 15 to 45% B over 20 min and then from 45 to 95% B over 3 min, which was maintained at 95% B for 2 min and the system was returned to 15% B over 2 min. The final conditions were maintained for 8 min before subsequent injections. The flow rate was 1 ml/min, and the column temperature was 25 ºC. We altered the UV detection wavelength from the original conditions used in Hurley et al. (1999) (300 nm) to increase the sensitivity of the methodology. We set UV detection at 270 nm, which is the wavelength corresponding to the maximum absorption of the tripeptide. The results were expressed as nmol of product per g of sample (dry matter) per hour.

Because plasmin activity has been reported to change with scalding temperature in some cheeses, we also assessed plasmin activity in the cheeses at 90 d of ripening using the method of Richardson & Pearce (1981).

***Proteolysis Assessment***

Proteolysis was assessed on 6-, 50- and 90-day-old cheeses by the techniques described below.

***Soluble Nitrogen.*** The cheese samples were treated to obtain a crude citrate extract from which soluble fractions at pH 4.6 (SN-pH 4.6), in 0.73 mol/l trichloroacetic acid (SN-TCA) and in 0.009 mol/l phosphotungstic acid (SN-PTA) were prepared (Gripon et al.

1975; Hynes et al. 2003). The nitrogen content in each fraction was determined by the macro-Kjeldhal method (IDF, 1993).

***Electrophoresis.*** The insoluble residue at pH 4.6 was analysed by urea-PAGE in a Mini-Protean II electrophoresis unit (BioRad Laboratories, Hercules, CA) according to the method of Andrews (1983). The proteins were stained with Coomassie Blue G-250. The different bands were visually evaluated.

***Peptide Profiles in Cheeses by RP-HPLC.*** The HPLC equipment and the chromatographic column were the same as in the analysis of the residual coagulant activity. Sample preparation and conditions of chromatographic analysis were made according to Hynes et al (2003).The different profiles were visually compared.

***Statistics***

Fat matter, protein content, moisture and plasmin activities were processed by two-way analysis of variance (ANOVA). pH, residual coagulant activity and soluble nitrogen were processed as describe below. The time of ripening, coagulant type and scalding temperature were selected as the main fixed factors for analysis. The analysis of variance on the variables measured during ripening used a repeated measurements model (PROC MIXED, SAS Ver. 9.2, SAS Institute, Inc., Cary, NC, USA). Significant interaction effects ($P < 0.05$) were handled by analysing the effect (main or interaction) of the involved treatment factor separately at each time point within the non-reducible model, using the technique of slicing (PROC MIXED, SAS Ver. 9.2). A least square difference (LSD) test was used as the post-hoc test for pair-wise comparisons in all ANOVA models ($\alpha = 0.05$). The normality of data distribution was tested on the model residuals from the Shapiro - Wilks test, besides qq plot construction.

## Results

### *Gross Composition and pH of Cheeses*

The gross composition of all cheeses was similar. The means and standard deviations of the fat and total protein in the dry matter, and moisture of cheeses were 38.90 ± 0.83, 54.32 ± 0.47 g/100g and 38.10 ± 1.17 g/100g, respectively. The pH increased during storage, regardless of coagulant and scalding temperature (Table 1). Statistically significant differences were detected between the pH at 90 days and the remainder of the times recorded (table 1 and 2).

### *Residual Coagulant and Plasmin Activity*

The residual coagulant activity in the cheeses is shown in Table 1. The cheeses scalded at 50 °C had a significantly higher residual coagulant level than the cheeses cooked at 56 °C, which was tested over the entire ripening process (Table 2). However, the coagulant activity increased significantly during the ripening of all cheeses, which suggested reactivation of the enzyme after heat treatment. This effect was more evident for the cheeses scalded at 56 ºC, and consequently, the differences in the residual coagulant activity between cheeses scalded at 56 ºC and 50 ºC were lower at the end of the ripening than at the beginning (Table 1). However, the interaction scalding temperature × ripening time was not significant.

Regarding the type of coagulant used, the differences between residual CH and CA were not statistically significant in Reggianito cheeses, regardless of scalding temperature and ripening time (Table 2). These results indicate that the experiments for the selection of equivalent doses of both coagulants were accurate not only to obtain equal milk-clotting times in cheese making but also to provide similar activities in the cheese during ripening.

The plasmin activity of 90-d-old cheeses was not affected by cooking temperature. The mean value for all cheeses was 4.65 ± 1.13 nmoles AMC $min^{-1}$ $g^{-1}$.

***Proteolysis Assessment***

***Soluble Nitrogen.*** The nitrogen content in the different soluble fractions (SN) increased significantly during cheese ripening (Table 2). Moreover, the coagulant type and the scalding temperature had a significant influence in the SN fractions. Overall, the CA cheeses showed significantly lower levels than the CH cheeses at the two scalding temperatures tested during the entire ripening process (Table 1). For the SN-TCA fraction, a significant interaction between the coagulant and temperature factors was found (Table 2), however the difference between the CA and CH cheeses was only significant in those scalded at 56 °C. On other hand, clear differences in the values of all the nitrogen fractions were detected and were dependent on the intensity of the heat treatment applied during scalding. These differences were consistent over the ripening period for both coagulants tested, with significantly lower values for the cheeses cooked at 56 °C (Table 1). For SN-TCA, the scalding temperature also significantly interacted with the ripening time (Table 2). While the values of SN-TCA increased significantly during ripening time for the two temperatures used, the change was lower at 56 ºC than at 50 ºC. At 6 days of ripening, the SN-TCA level was similar in all cheeses, but the differences increased with ripening time. Interestingly, the differences in proteolysis that are attributable to scalding temperature were higher for the cheeses made with camel coagulant. For example, the increase in nitrogen content in the soluble fractions of the CA-50°C cheeses compared to CA-56°C was higher than the difference between the respective CH cheeses (Table 1).

298 ***Electrophoresis.*** The electrophoretic profiles of the pH 4.6-insoluble N fractions of the
299 cheeses are shown in Figure 1. As observed for the SN fractions, the CA cheeses showed
300 lower levels of proteolysis than CH at the two scalding temperatures tested. At 6 days of
301 ripening, a weak band of $\alpha_{s1}$-I (f24-199) was observed in the cheeses made with CH,
302 which was not found in the CA cheeses. Concomitantly, a lower intensity for the intact
303 $\alpha_{s1}$-casein band was found in the CH cheeses.
304 Regarding the influence of temperature, the $\alpha_{s1}$-I fraction was detected in the 50°C CA
305 and CH cheeses from the beginning of ripening, while for those scalded at 56 °C, it was
306 observed only after 50 days. Similarly, the intensity of this fraction at 50 and 90 days was
307 higher in the cheeses with the lower scalding temperature. In contrast, the γ-casein
308 fractions increased during ripening but there were no differences between the cheeses
309 with different scalding temperatures or coagulants. There were also no effects observed
310 for the band corresponding to β-casein for neither temperature nor type of coagulant.
311
312 ***Peptide Profiles.*** During ripening, the changes in the peptide profiles of each type of
313 cheese were recorded, and these changes were more evident in those scalded at 50 °C
314 (Figure 2). In the 50 °C cheeses, a marked decrease of the hydrophobic peptides, found
315 in the middle and final parts of the profiles (at elution times higher than 40 min), and a
316 greater increase of the hydrophilic peptides at the beginning of the chromatogram, was
317 observed in comparison with cheeses scalded at 56 ºC. When both coagulants were
318 compared, the profiles were similar for the samples scalded at 50 °C, while minor
319 differences were found for the cheeses scalded at 56 °C (Figure 2 A and B). In the last
320 samples, less proteolysis was detected in the CA cheeses, which was evidenced by lower
321 heights of the initial peaks.
322

## Discussion

Proteolysis during cheese ripening depends on the active proteolytic agents and the environmental conditions in the food matrix, which are determined by the cheese making technology. In the present work, we investigated the effect of lowering the curd scalding temperature in Reggianito cooked cheese to increase proteolysis and promote accelerated ripening. The cheeses were made with two coagulant enzymes that are widespread in Argentina: bovine and camel chymosins.

During cheese making, the amount of coagulant retained in the curd depends on several factors, such as pH at whey draining, scalding temperature, cheese moisture and type of coagulant (Bansal et al. 2007; Bansal et al. 2009b). Consequently, the residual coagulant activity varies widely among cheese varieties (Bansal et al. 2009b; Grappin et al. 1985). In this work, no changes in the moisture content of the cheeses were obtained, even for different cooking temperatures. This was achieved by working the mixture of whey and curds for longer during the "curd drying" step, i.e., below 45 °C, and it allowed us to compare the residual coagulant amount and proteolysis during ripening in similar cheese matrices.

Cooked cheeses are not generally considered to contain significant residual coagulant activity (Garnot & Mollé, 1987; Delacroix-Buchet & Fournier 1992; Rampilli et al. 1998). However, our results showed that coagulant inactivation is not complete, and more interestingly, that an increase of the enzyme occurred during ripening. The increase was more pronounced when scalding was conducted at 56 °C. A similar trend was previously reported for Reggianito cheeses cooked at different temperatures, which included a scalding step as high as 60 ºC (Hynes et al. 2004). Previous investigation also revealed that chymosin inactivation was a partial and reversible process in Cheddar cheeses (Hayes

et al. 2002). In the present work, we detected that the enzyme activity at 90 days of ripening was approximately 2 times higher than the initial level in the cheeses scalded at 50 ºC, while a 4-fold increase was found in the cheeses cooked at 56 ºC. Consequently, the coagulant activity levels between the 90-day-old cheeses were not as different as in the 6-day-old cheeses.

In previous works, it was demonstrated a null thermostability of several chymosin (bovine, camel, buffalo and goat) after 24 h of treatment at temperatures above 50ºC. However, when the heat treatment was applied during 1 h, some residual activity was detected (Vallejo et al. 2012). In the cheeses described in our work, the heat treatment was applied only during a few minutes during cheese making, so it is possible that the inactivation effect was limited. On other hand, Garnot & Mollé (1987) verified that the most of chymosin in milk was inactivated in conditions encountered during Swiss cheese making (53ºC at pH 6.5 during 60 min) and showed a lower inactivation when the pH was 6.50 in comparison with 6.75. In our work, the pH during milk coagulation was lower (6.30). Meanwhile, Delacroix-Buchet & Fournier (1992) found that the production of the peptide $\alpha_{s1}$-I from $\alpha_{s1}$ casein in Gruyère cheeses was retarded during ripening when the cooking temperature was raised from 52 to 56ºC, and they suggested a higher inactivation of chymosin at the higher cooking temperature.

For the source of the chymosin, our results indicated that both enzymes had similar stability towards thermal treatment, which was demonstrated by the residual coagulant activity. However, certain proteolysis indexes, such as the peptide profiles and SN-TCA, indicated significantly lower proteolysis in the cheeses made with camel chymosin when the highest scalding temperature was applied. These results suggested that camel chymosin is more heat labile when a cooking temperatures of 56 °C was applied. Contradictory results were found by Vallejo et al. (2012), who reported that camel

chymosin was more thermostable than bovine chymosin between 50 and 60 ºC. However, in this work, the thermostability of the chymosins was assessed in assays carried out in buffer solutions, and the effect of cheese matrix on this property was not considered. In this sense, it is important to take into account that different milk compounds have a protective effect on the stability of chymosin, as was verified in previous works (Garnot & Mollé 1987).

The curd scalding temperature may affect plasmin activity (Ismail & Nielsen, 2010, Somers & Kelly, 2002); therefore, we monitored the level of this indigenous enzyme in our cheeses. No significant differences were found, which is consistent with previous studies on Reggianito-type mini-curds (Velez et al. 2011). The differences in plasmin were only significant in the cheeses cooked at very different temperatures, with changes from 48 to 55 ºC for the scalding conditions (Somers & Kelly, 2002). In addition, higher levels of plasmin were observed in Swiss cheese, which is cooked at 50 ºC, compared with Cheddar cheese, where the cooking temperature during production is 37 ºC (Ismail & Nielsen, 2010).

$\alpha_{s1}$-Casein is the most susceptible protein in the cheese matrix to the activity of coagulant enzymes during cheese ripening, and the primary site of attack is the $Phe_{23}$-$Phe_{24}$ bond to subsequently produce of peptides $\alpha_{s1}$ (f1-23) and $\alpha_{s1}$-I (f24-199) (Carles & Ribadeau-Dumas, 1985). The peptide $\alpha_{s1}$-I (f24-199), is water-insoluble and can be observed in the electrophoretic study (Fox, 2003; McSweeney, 2004). Our results for the electrophoretic profiles corroborate the residual coagulant activity and the SN fractions. The cheeses scalded at 50 °C that showed an increased residual coagulant activity also showed higher $\alpha_{s1}$-casein degradation with a consequent higher production of peptide $\alpha_{s1}$-I (f24-199). Similar results were obtained by Sheehan et al. (2007), who observed that an increase in

397 the scalding temperature during semi-hard cheese making lowered the $\alpha_{s1}$-casein
398 degradation in the 53 < 50 < 47 ºC range.
399 For the soluble fractions, the same trend was found, where there was increased N content
400 in all fractions assayed for cheeses scalded at 50 °C. In SN-pH 4.6 and SN-TCA, this
401 increase was most likely due to the direct influence of coagulant activity, which provided
402 large and intermediate peptides. Higher levels of SN-PTA during ripening also may
403 reflect an indirect influence of the coagulant because a richer pool of intermediate size
404 peptides that most likely favored proteolysis and peptidolysis due to the starter culture
405 (Sousa et al. 2001; Gobetti & DiCagno, 2003; Upadhyay et al. 2004).
406 Although the increased coagulant activity and the production of its typical derived
407 peptides are consistent in our work, contribution of proteolytic agents other than
408 chymosin should not be discarded. Cathepsin D (Hayes et al., 2001) and cell envelope
409 proteinases of starter cultures of *Lactobacillus helveticus* may contribute to $\alpha_{s1}$-I
410 production in cheeses cooked at high temperatures (Delacroix-Buchet & Fournier 1992;
411 Sadat-Mekmene et al. 2013).
412 For proteolysis related to the type of chymosin, the camel coagulant showed slightly
413 lower proteolytic activity during ripening, which was verified by lower levels of SN
414 fraction and lower heights of the initial peaks in the peptide profile at 56 °C in the cheeses
415 produced with camel chymosin in our study. A similar trend was obtained by Bansal et
416 al. (2009a) in Cheddar cheeses produced with the same enzymes. Nevertheless, these
417 authors found more pronounced differences in the peptide profiles of the Cheddar cheeses
418 analysed, attributing such differences both to the lower amount of camel coagulant they
419 added and to its less intensive nonspecific proteolytic activity. Camel chymosin has been
420 reported to have highly specific coagulant activity and low nonspecific proteolytic
421 activity, which outperforms bovine chymosin (Kappeler et al. 2006). Bansal et al. (2009a)

422 also found β-casein hydrolysis in cheeses made with bovine chymosin, which was not
423 exhibited in camel chymosin cheeses. In our study, however, we did not identify β (f1-
424 189/192) in any of the cheeses analysed. The changes attributable to the type of chymosin
425 were detected in our work in the soluble nitrogen fractions and by the peptide profiles.
426 In low-fat and reduced-fat Cheddar cheese, the use of camel chymosin in the cheese
427 making process resulted in lowered proteolysis and less bitterness, an effect that was
428 attributed to a decreased production of hydrophobic peptides from β-casein
429 (Govindasamy-Lucey et al. 2010; Børsting et al. 2012). Møller et al. (2012) also verified
430 lower proteolytic activity of camel chymosin on $\alpha_{s1}$ and β-casein in vitro in comparison
431 with bovine chymosin. Møller et al. (2013) found that low salt Cheddar cheeses made
432 with bovine chymosin contained lower amounts of intact $\alpha_{s1}$-casein than those made with
433 camel chymosin, especially during early ripening. In low-moisture, part skim mozzarella
434 cheese, Moynihan et al. (2014) also found lower proteolysis in cheeses manufactured with
435 camel chymosin than in cheeses made with bovine chymosin, which the authors deemed
436 beneficial for longer shelf-life of the resultant mozzarella. As in our study, Moynihan et
437 al. (2014) used equivalent levels of both coagulants that were calculated based on the
438 milk clotting time.
439 Thus far, most reported investigations on the use of camel chymosin indicate that defects
440 attributable to bovine chymosin-mediated proteolysis, especially in low fat or part skim
441 cheeses, can be avoided or minimized by replacing it with camel chymosin. However, in
442 our study, we did not find defective or negative proteolytic changes associated with
443 proteolysis by bovine chymosin, and its replacement by camel chymosin did not
444 significantly change the primary proteolysis pattern of the cheese. In effect, there were
445 no changes observed for the hydrolysis products of β-casein according to the type of

coagulant. Neither difference in hydrophobic region of peptide profiles – above 40 min retention time - were observed when both coagulants were compared.

The investigation of cheese proteolysis mediated by camel chymosin is new and is most likely not yet conclusive, especially when different cheeses are compared.

## Conclusions

Accelerated proteolysis was achieved in Reggianito cheeses when a lower scalding temperature was applied, due to increased retention of coagulant activity. The residual coagulant activity increased during ripening and tended to equalize in all cheeses at 90 days. However, the residual activity, as well as the proteolysis, was always higher in the cheeses scalded at the lower temperature by all applied indexes.

For the comparison between camel and bovine chymosin, our results confirm that camel chymosin is less proteolytic than bovine chymosin, although the differences were not as marked as those published by other authors. The proteolysis patterns were similar for both enzymes, especially as observed by electrophoresis, but the bovine chymosin showed a more rapid progression of proteolysis during the ripening for all samples. No evidence of defective or unexpected proteolysis products, as hydrophobic peptides derived from β-casein, was detected in either cheese.

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655 **Table 1.** pH, residual coagulant activity (nmol product $h^{-1}$ $g^{-1}$) and SN fractions in cheeses
656 expressed as the percentage of total N during ripening (means ± standard deviation of
657 duplicate analysis of the four replicas of cheeses). For chymosin activity, the highlighted
658 results shown that the coagulant activity at the end of ripening was higher than in the
659 young cheeses

| | | Cheeses | | | |
|---|---|---|---|---|---|
| | | CA | | CH | |
| Item | Ripening time | 50 ºC | 56 ºC | 50 ºC | 56 ºC |
| pH | 6 | 5.15 ± 0.05 | 5.23 ± 0.04 | 5.21 ± 0.01 | 5.23 ± 0.01 |
| | 50 | 5.20 ± 0.14 | 5.28 ± 0.04 | 5.24 ± 0.01 | 5.21 ± 0.07 |
| | 90 | 5.29 ± 0.13 | 5.41 ± 0.08 | 5.31 ± 0.05 | 5.36 ± 0.06 |
| Chymosin activity | 6 | 183.8 ± 46.8 | 63.2 ± 28.3 | 124.2 ± 15.8 | 56.8 ± 39.3 |
| | 50 | 269.8 ± 64.1 | 132.1 ± 34.4 | 194.0 ± 74.6 | 169.9 ± 30.6 |
| | 90 | 321.6 ± 48.2 | 276.1 ± 30.4 | 326.4 ± 66.8 | 249.2 ± 35.6 |
| SN-pH 4.6 | 6 | 5.0 ± 0.6 | 3.1 ± 1.5 | 5.0 ± 0.2 | 4.7 ± 0.1 |
| | 50 | 7.7 ± 0.4 | 6.1 ± 0.9 | 9.2 ± 0.8 | 6.6 ± 0.2 |
| | 90 | 8.9 ± 0.2 | 7.8 ± 1.9 | 10.5 ± 0.7 | 10.2 ± 1.7 |
| SN-TCA | 6 | 2.8 ± 0.3 | 1.1 ± 0.1 | 3.0 ± 0.1 | 2.4 ± 0.1 |
| | 50 | 6.7 ± 0.3 | 2.7 ± 0.8 | 7.0 ± 0.5 | 5.2 ± 0.6 |
| | 90 | 7.7 ± 0.1 | 4.9± 1.1 | 8.7 ± 0.3 | 6.4 ± 0.1 |
| SN-PTA | 6 | 1.4 ± 0.1 | 0.8± 0.4 | 1.3 ± 0.1 | 1.0 ± 0.1 |
| | 50 | 3.2 ± 1.0 | 1.7 ± 0.3 | 3.3 ± 0.4 | 2.2 ± 0.1 |
| | 90 | 4.5± 0.7 | 2.5± 0.7 | 5.6 ± 0.1 | 3.4 ± 0.1 |

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**Table 2.** Significance of effects time of ripening (F1), coagulant type (F2) and scalding temperature (F3) and their interaction‡ on pH, chymosin activity and SN fraction

| | Significance | | | | | | |
|---|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F1xF2 | F1xF3 | F2xF3 | F1 x F2 x F3 |
| **pH** | * | NS | NS | NS | NS | NS | NS |
| **Chymosin activity** | * | NS | * | NS | NS | NS | NS |
| **SN-pH 4,6** | * | * | * | NS | NS | NS | NS |
| **SN-TCA** | * | * | * | NS | * | * | NS |
| **SN-PTA** | * | * | * | NS | NS | NS | NS |

‡ * P<0.05. NS: no significant.

684 **Figure captions**

685 **Figure 1**. Electrophoretic profiles of insoluble cheese fractions at pH 4.6 during ripening.

686 **Figure 2.** Peptide profiles of water-soluble extracts of cheeses at 6, 50 and 90 days of

687 ripening. **A)** CA cheeses at 50 and 56 ºC, **B)** CH cheeses at 50 and 56 ºC.

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690 **Figure 1**

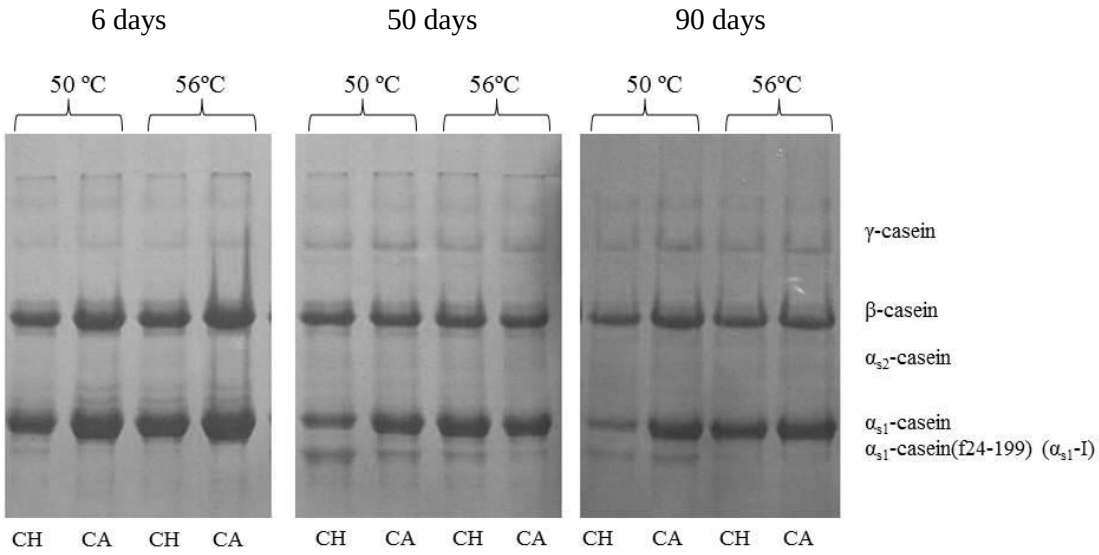


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**Figure 2.**