



Effect of high-pressure treatment on hard cheese proteolysis

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ABSTRACT

The application of high hydrostatic pressure (HHP) treatment has been proposed to reduce the ripening time of cheese via modifications in the enzymatic activities or the substrate reactivity. Investigations on the effect of HHP on cheese proteolysis have been undertaken with either encouraging results or little effect according to the treatment conditions and the type of cheese, but information concerning the effect of HHP on the ripening of hard cooked cheese is still lacking. In this report, we describe the effect of HHP treatment on Reggianito cheese proteolysis. For that purpose, 1-d-old miniature cheeses (5.5-cm diameter and 6-cm height) were treated at 100 or 400 MPa and 20°C for 5 or 10 min, and control cheeses in the trial were not pressurized. All cheeses were ripened at 12°C during 90 d. The HHP did not affect gross composition of the cheeses, but microbial load changed, especially because the starter culture count was significantly lower at the beginning of the ripening of the cheeses treated at 400 MPa than in controls and cheeses treated at 100 MPa. Cheeses treated at 400 MPa for 10 min had significantly higher plasmin activity than did the others; the residual coagulant activity was not affected by HHP. Proteolysis assessment showed that most severe treatments (400 MPa) also resulted in cheeses with increased breakdown of α_{S1} - and β -CN. In addition, nitrogen content in soluble fractions was significantly higher in cheeses treated at 400 MPa, as well as soluble peptides and free AA production. Peptide profiles and individual and total content of free AA in 60-d-old treated cheese were as high as in fully ripened control cheeses (90 d). Holding time had an effect only on pH-4.6-soluble nitrogen fraction and plasmin activity; cheese treated for

10 min showed higher values than those treated for 5 min, at both levels of pressure assayed. We concluded that HHP treatments at 400 MPa applied 1 d after cheesemaking increased the rate of proteolysis, leading to an acceleration of the ripening process in Reggianito Argentine cheese, whereas 100-MPa treatments did not lead to significant changes.

Key words: high hydrostatic pressure, Reggianito cheese, ripening acceleration, proteolysis

INTRODUCTION

High hydrostatic pressure (HHP) treatment, a non-thermal method of food preservation used for a wide range of products, has similar benefits to thermal treatment in food safety while causing minimal changes in food quality (Torres and Velazquez, 2005). Research into the application of HHP processing dates back to the end of the 19th century when Hite (1899) demonstrated that the shelf life of milk could be extended by high-pressure treatment. However, the unavailability of suitable equipment hampered further research for the first half of the 20th century. The first HHP-treated products appeared on the market in Japan in 1991 (Cheftel, 1995). In milk and dairy products, the current state of knowledge on the subject is primarily derived from research done since 1990. In recent years, interest in the effect of HHP treatment on cheese has increased. The main objective is to accelerate cheese ripening and reduce the ripening time, during which the biochemical transformations of proteins, fats, and carbohydrates that will result in the texture, flavor, and functionality of different cheeses will proceed (Trujillo et al., 2000; O'Reilly et al., 2001).

The potential use of HHP treatment for the acceleration of cheese ripening was first studied by Yokoyama et al. (1992). These researchers claimed that cheese exposed to HHP treatment at 50 MPa for 3 d at 25°C had characteristics typical of a 6-mo-old commercial Cheddar cheese. However, further studies in Cheddar

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(O'Reilly et al., 2000, 2001), Gouda (Messens et al., 1999), and goat cheese (Saldo et al., 2001) generally concluded that HHP treatments similar to those used by Yokoyama et al. (1992) had only minor effects on cheese ripening. These contradictory results were observed because the levels of starter bacteria added to the cheese milk by Yokoyama et al. (1992) were at least 10-fold higher than those used in the other studies, which probably influenced the acceleration of cheese ripening.

O'Reilly and colleagues studied the effect of different pressure ranges (50 to 400 MPa) and times both long (of the order of 3.5 and 81.5 h) and short (under 20 min) in Cheddar cheese (O'Reilly et al., 2000, 2002, 2003). Overall, these researchers determined that whereas cheese treated at low pressures and long holding times (~50–200 MPa during up to 82 h) had increased levels of primary proteolysis, HHP treatment at higher pressures for relatively shorter processing times (200–400 MPa for ~20 min) produced changes primarily in the protein structure, which improved the functional properties. These authors also found that the time during ripening in which the HHP treatment was applied also influenced the results, exhibiting stronger effects when the HHP treatment was applied to 1-d-old cheeses than to 15-d-old cheeses. Also for Cheddar cheese, studies performed by Rynne et al. (2008) using a pressure level of 400 MPa for 10 min had little effect on primary proteolysis compared with control cheeses. Ozturk et al. (2013a) studied the effect of pressure levels between 50 and 400 MPa and holding times of 5 and 20 min in reduced-fat Cheddar cheese. These researchers concluded that HHP treatment did not change the rate of proteolysis but improved the texture of the cheese. No change in the indexes of proteolysis was encountered when a treatment of 405 MPa for 3 min was applied on regular and reduced, low, and no salt added Cheddar cheeses, but certain microbiological, textural, and rheological properties were affected by HHP (Ozturk et al., 2013b). Similar results in proteolysis were observed in Edam cheese, where a pressure level between 200 and 500 MPa for a holding time of 30 min did not alter the rate of proteolysis, but treated cheese had an improvement of the consistency compared with control cheeses (Iwaczak and Winiewska, 2005). In a hard uncooked cheese, in which pressure levels between 200 and 500 MPa were applied for 10 min, an increase in the rate of proteolysis at 300 MPa was observed (Yang et al., 2014). In Mozzarella cheese, HHP treatment at 400 MPa for 5 min had no effect on the composition and functional properties (Sheehan et al., 2005). Studies in hard goat cheese treated at 400 MPa showed an increase in the rate of proteolysis and changes in the composition of the HHP-treated cheese with respect to

controls, although no change in plasmin activity and a decrease in the activity of the residual coagulant were observed (Saldo et al., 2002). In sheep cheeses, HHP treatment at 300 MPa for 10 min 1 d after manufacture resulted in an increase in the rate of proteolysis, higher levels of free AA (**FAA**), and an improvement in the texture of the cheese compared with controls (Juan et al., 2008). Brie cheese and blue-veined cheese, treated at 400 and 600 MPa for 5 and 10 min, respectively, exhibited a decrease in microbial counts compared with the control cheese (Voigt et al., 2010; Calzada et al., 2014). In addition, these researchers observed decelerated proteolysis of HHP-treated cheeses.

Different combinations of pressure level and holding time have been applied in several types of cheeses. Regarding hard cheeses, most studies have investigated Cheddar cheese and hard sheep cheeses. However, hard cooked cheeses, such as Parmigiano Reggiano, Grana Padano, Sbrinz, or Reggiano, that are ripened for periods ranging from 6 mo to 2 yr have not been subjected to HHP to date. Technological approaches to accelerate the ripening of hard cooked cheeses is a topic of interest in dairy technology, as keeping large volumes of cheese for such a long period is both expensive and time consuming.

Reggiano is a hard cooked cheese derived from Italian Grana and Parmesan cheeses. This cheese has existed in Argentina since the late 19th century. Italian immigrants first produced Reggiano cheese, which has since diverged from the original varieties but is also different from Parmesan-like American or generic products (Zannoni et al., 1994). Reggiano has a higher moisture content (between 31.3 and 34.9%), smaller size (between 5 and 10 kg of weight), and shorter ripening period (minimum 6 mo) than Italian hard cheeses, who have a moisture between 29.9 and 31.7%, weight of 37 kg on average, and minimum ripening time of 12 mo (Zannoni et al., 1994; Sihufe et al., 2012). Reggiano cheese is the most important hard cheese variety manufactured in Argentina, and it is exported primarily to Brazil, Russia, the United States, Chile, and Venezuela.

In this work, we studied the effect of pressure level (100 and 400 MPa) and holding time (5 and 10 min) on Reggiano cheese proteolysis.

MATERIALS AND METHODS

Experimental Design

We assessed the influence of pressure and holding time on Reggiano cheese proteolysis. Both factors were studied at 2 levels (pressure: 100 and 400 MPa; holding time: 5 and 10 min), which accounted for 4 treatments: **T1** (100 MPa, 5 min), **T2** (100 MPa, 10 min), **T3**

(400 MPa, 5 min), and T4 (400 MPa, 10 min). Control cheeses (unpressurized) were also included. Four replicates of the cheese makings were performed, with different milk. Cheeses were pressurized on the first day after cheesemaking.

Cheesemaking

Experimental Reggiano Argentino cheeses were manufactured according to the standard process (Galino, 1994), using miniature molds. The use of miniature molds was needed to obtain miniature cheeses with optimum size for introduction into the vessel of the equipment used for HHP treatment. We made 4 replicate cheesemaking experiments using different milk batches, and on different days. Cow's milk was obtained from a dairy farm of Instituto Nacional de Tecnología Agropecuaria (Estación Experimental Agropecuaria Rafaela, Santa Fe, Argentina; mean compositional values \pm SD were as follows: $3.62 \pm 0.06\%$ fat; $3.50 \pm 0.07\%$ protein; $12.68 \pm 0.06\%$ total solid; pH 6.81 ± 0.06). Cheesemaking was performed in a 180-L vat provided with automated variable-speed cutting and stirring devices (Industria Metalúrgica en Acero Inoxidable, IMAI, Rafaela, Santa Fe, Argentina). After adjusting fat content to a fat-protein ratio of 0.7, milk was batch pasteurized at 65°C for 20 min and cooled to 32°C , and CaCl_2 was added at a final concentration of 0.02% (wt/vol). Milk pH was adjusted to the target value for Reggiano cheeses (6.25 ± 0.02) with lactic acid (1.5% vol/vol); after that a commercial starter composed of mixed strains of *Lactobacillus helveticus* (Chr. Hansen Argentina, Quilmes, Argentina) was added in a concentration of 10^6 cfu/mL of milk. Milk clotting enzyme was pure chymosin (Chy-Max, Chr. Hansen Argentina, 570 international milk clotting units/mL); it was added to the milk immediately after the starter at a concentration of 0.06 mL/L of milk. Next, a hot wire viscosity sensor (INRA, Pignat, France) was introduced into the vat, and the coagulation time was detected using de Coag software (INRA). This sensor measures the time between the coagulant addition and the beginning of milk clotting. The coagulation time was 7.18 ± 0.66 min (mean value \pm SD), after which the curd was allowed to strengthen up for 2.16 ± 0.20 min, and then it was subsequently cut to the adequate grain size (approximately 1–3 mm) with an automated device for curd cutting and stirring coupled to the vat. The mixture of the curd particles and whey was gently stirred and heated at $0.5^\circ\text{C}/\text{min}$ until it reached 42°C to reduce moisture in curd grains, and it was subsequently held at this temperature for 15 min. The mixture was then more rapidly heated to 51°C ($1^\circ\text{C}/\text{min}$). When this temperature was reached, the mixture of whey and

curds was kept for 3 min (cooking step). After that, the curds were separated from the whey and placed in miniature cylindrical molds that were 5.5 cm in diameter and 10 cm in height. From each vat, 40 minicheeses were obtained. The average cheese weight was 250 g. Next, cheeses were pressed at room temperature (20°C) in a horizontal press for 20 h and brined for 6 h in saturated brine at 12°C . After that, minicheeses were vacuum packed using Cryovac BB2800CB bags (permeability to O_2 at $3 \times 10^{-4} \text{ cm}^3 \text{ m}^{-2} 24 \text{ h}^{-1} \text{ Pa}^{-1}$; CO_2 at $15 \times 10^{-4} \text{ cm}^3 \text{ m}^{-2} 24 \text{ h}^{-1} \text{ Pa}^{-1}$; and water vapor at $20 \text{ g} \cdot 24 \text{ h}^{-1} \cdot \text{m}^{-2}$; Sealed Air Co., Buenos Aires, Argentina). The 40 cheeses obtained by cheesemaking were randomly divided in control, T1, T2, T3, and T4 (8 for each treatment, including control).

High-Pressure Processing

Vacuum-packed 1-d-old cheeses were subjected to HHP treatment, applied in a High Pressure System Stansted Fluid Power Ltd. model Iso-Lab FPG9400:922 (Stansted, United Kingdom), with a vessel working volume of 2 dm^3 (maximum working pressure: 900 MPa; temperature range: -20 to 120°C). A mixture of propylene glycol and water (30:70) was used as compression fluid. Pressurization rate was 300 MPa/min. The conditioning temperature of vessel and initial temperature of compression fluid was 20°C . The adiabatic heating induced an increase of temperature that reached a maximum at 400 MPa. In this case, the fluid temperature increased up to 30°C at the end of the compression stage. After HHP treatment, the packages of the cheese were removed, and all cheeses (pressurized and control) were ripened 90 d at 85% relative humidity and 12°C . After the first 2 wk of ripening, all cheeses were vacuum packed again with the same bags described above to avoid over drying. This time was selected according to a previous experiment for the validation of the model (Vélez, 2013), in which it was assessed that the moisture content of the miniature cheeses reached levels of 34 to 35%, similar to commercial Reggiano cheese at the end of ripening, after 2 wk of ripening. One cheese of each treatment, including control cheese, was selected randomly on d 1, 45, and 90 after treatments and was completely grated. Then, grated cheese samples were taken and were immediately analyzed for gross composition and pH or stored at -18°C for further analysis.

Cheese Analysis

All assays were performed in duplicate at 1, 45, and 90 d after HHP treatment.

Gross Composition of Cheeses and Microbiological Counts. Total protein, fat, and DM were assessed according to International Dairy Federation standards (IDF, 1982, 1993, 1997). The results of total protein and fat were expressed in DM. The pH was determined as described by Bradley et al. (1993) by immersing of the electrode (Schott Instrument, Mainz, Germany) in a homogenate (1:1) of grated cheese thoroughly mixed with distilled water.

Mesophilic and thermophilic lactobacilli were enumerated by counting the colonies on de Man, Rogosa, and Sharpe (MRS) agar plates (Laboratorios Britania, Buenos Aires, Argentina) after incubating the plates at 30 and 45°C, respectively, for 48 h under microaerophilic conditions (Corsetti et al., 1998).

Residual Coagulant and Plasmin Activity. The residual coagulant activity in the cheese samples was assessed according to the method of Hurley et al. (1999), with some modifications as described by Costabel et al. (2015). This method quantifies the hydrolysis of a synthetic chromophoric heptapeptide substrate [Pro-Thr-Glu-Phe-(NO₂-Phe)-Arg-Leu; Bachem California Inc., Torrance, CA]. From this substrate, aspartic proteases are able to release the tripeptide [(NO₂-Phe)-Arg-Leu] in proportion with their activity; this tripeptide is then quantified by reverse-phase HPLC (RP-HPLC). The HPLC system consisted of a quaternary pump, an online degasser, and a UV/visible detector (all Series 200, Perkin Elmer, 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). The results were expressed as nanomoles of product per gram of sample (DM) per hour.

Plasmin activity in the cheeses was assessed using the method of Richardson and Pearce (1981). Briefly, this method assesses enzymatic activity on a nonfluorescent substrate, succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-coumarin (Sigma-Aldrich, St. Louis, MO), which results in the fluorescent product 7-amido-4-methyl-coumarin (AMC) after hydrolysis. The AMC production was detected by measuring fluorescence with a spectrofluorometer (Hitachi f-2000, Tokyo, Japan) for 30 min at 5 min intervals. The plasmin activity in the cheeses was calculated with a calibration curve obtained with known AMC concentrations and was defined as nanomoles of AMC released per gram (of DM) per minute at pH 7.5 and 25°C.

Proteolysis Assessment. Proteolysis was assessed by the techniques described below.

The cheese samples were treated to obtain a crude citrate extract from which soluble N fractions at pH 4.6

(SN-pH 4.6) and in 12% (wt/vol) trichloroacetic acid (SN-TCA) were prepared (Gripon et al., 1975; Hynes et al., 2003). The N content in each fraction was determined by the macro-Kjeldahl method (IDF, 1993).

The insoluble residue at pH 4.6 was analyzed by urea-PAGE in a Mini-Protean II cube (BioRad Laboratories, Hercules, CA) according to the method of Andrews (1983). The proteins were stained with Coomassie Blue G-250.

The HPLC equipment and the chromatographic column were the same as in the analysis of the residual coagulant activity. The water-soluble extracts of the cheeses were obtained, filtered through 0.45- μ m membranes (Millex, Millipore, São Paulo, Brazil), and injected into the HPLC instrument. Detection was performed at 214 nm, the column temperature was 40°C, and the flow rate was 1 mL/min. Separation was achieved using an increasing linear gradient of acetonitrile in water over 107 min (Hynes et al., 2003).

Individual FAA were determined on 12% (wt/vol) trichloroacetic acid-soluble filtrates prepared from SN-pH 4.6 fraction according to the method described by Cattaneo et al. (2014). Next, the pH of 10 mL of the FAA extract was adjusted to 2.2 with NaOH and filtered using a syringe filter with a 0.2-mm membrane. The FAA concentrations were determined using a Biochrom 30 series AA analyzer (Biochrom US, Holliston, MA). The light absorption was measured at 570 nm. Calibration curves were obtained using an AA standard purchased from Sigma (18AA-Sigma). For quantification, peak areas were correlated with the concentrations according to calibration curves. The results were expressed as milligrams of AA per 100 g of cheese (DM).

Statistics

The time of ripening and treatment applied were selected as main factors for the analysis. The ANOVA on the variables measured during ripening used a repeated measurements model (PROC MIXED, SAS Ver. 9.2, SAS Institute Inc., Cary, NC). Significant interaction effects ($P < 0.05$) were handled by analyzing the effect (main or interaction) of the involved treatment factor separately at each time of ripening point within the nonreducible model using the technique of slicing (PROC MIXED, SAS Ver. 9.2). Least square difference test was used as a post hoc test for pair-wise comparisons in all ANOVA models ($\alpha = 0.05$).

Peptide profiles were analyzed by multivariate methods, including a fuzzy logic approach for data preprocessing for RP-HPLC chromatograms of peptide profiles (Piraino et al., 2004). Next, principal components analysis was applied to reduce dimensionality of peptide profiles. Principal components analysis was

performed using the SPSS 10.0 software (SPSS Inc., Chicago, IL).

RESULTS

Cheese Composition and pH

The HHP treatments of cheeses 1-d after cheesemaking did not significantly affect their gross composition and pH, as measured at 1, 45, and 90 d after HHP treatment. The time of ripening affected the pH and moisture content (Table 1). The pH increased and the moisture decreased significantly ($P < 0.05$) during storage of all cheeses (control and HHP treatments). The decrease of moisture was expected because of water evaporation before vacuum packing of cheeses.

Microbiological Counts

Thermophilic lactobacilli (i.e., microbial counts on MRS at 45°C), which represent mainly the starter of *Lactobacillus helveticus*, decreased significantly ($P < 0.05$) in all cheeses during ripening time (Table 2). A significant interaction ($P < 0.05$) between the HHP treatment and the time of ripening was observed (Table 1). On d 1 after HHP treatment, microbial counts enumerated on MRS at 45°C were significantly lower ($P < 0.05$) in T3 and T4 cheeses than in control, T1, and T2 cheeses (Table 2), which suggested an effect of the treatment on the viability of the starter culture. However, no significant differences were found between control and HHP-treated cheeses both at 45 and 90 d of ripening.

For microorganisms grown on MRS at 30°C, bacterial counts in both control and HHP-treated cheeses decreased significantly ($P < 0.05$) during the 90 d of the ripening period (Tables 1 and 2). A significant interaction ($P < 0.05$) between the HHP treatment and time of ripening was found (Table 1). For each time of ripening, a different trend was found. On d 1 after HHP treatment, microbial loads in T3 and T4 cheese were significantly ($P < 0.05$) lower than in the control and in T1 and T2 cheeses. An opposite trend was found after 45 and 90 d of ripening, with counts significantly ($P < 0.05$) higher for T3 and T4 than control, T1, and T2 cheeses (Table 2). Microbial counts on MRS at 30°C represent mainly mesophilic lactobacilli, but we considered that the thermophilic lactobacilli of the starter, whose optimal growth temperature is 45°C, were not completely inhibited at 30°C. So, at the end of ripening, as starter microorganisms decreased, probably the nonstarter lactic acid bacteria population was mainly enumerated in this condition.

Table 1. Significance of the effect of high hydrostatic pressure treatment on Reggianito cheese (F1), ripening time (F2), and their interaction on gross composition, pH, thermophilic and mesophilic lactobacilli, plasmin activity, coagulant activity, soluble nitrogen (SN) fraction, Asp, and total free AA

Item	Significance		
	F1	F2	F1 × F2
Protein in DM	NS	NS	NS
Fat in DM	NS	NS	NS
Moisture	NS	*	NS
pH	NS	*	NS
Thermophilic lactobacilli	*	*	*
Mesophilic lactobacilli	NS	*	*
Plasmin activity	*	NS	NS
Coagulant activity	NS	*	NS
SN-pH 4.6	*	*	NS
SN-TCA ¹	*	*	NS
AA Asp	*	*	NS
Total free AA	NS	*	NS

¹TCA = trichloroacetic acid.

* $P < 0.05$.

Plasmin and Residual Coagulant Activity

The activities of plasmin and residual coagulant enzyme in the cheeses are shown in Table 2. Although plasmin activity had a tendency to decrease with ripening time, differences were not statistically significant (Table 1). Regarding HHP treatment, cheeses treated at 400 MPa for 10 min had significantly ($P < 0.05$) higher plasmin activity than did the control and other HHP treatments. The residual coagulant activity increased significantly ($P < 0.05$) in all cheeses during ripening; therefore, HHP had no detectable effect in the level of residual coagulant activity.

Proteolysis Assessment

SN. The nitrogen content in the different soluble fractions (SN) increased significantly ($P < 0.05$) during cheese ripening (Table 1). Moreover, HHP had a significant ($P < 0.05$) influence on the 2 SN fractions assessed. In this sense, the levels of the SN-pH 4.6 fraction in T4 cheese were statistically the same as those in T3 cheese but different in control, T1, and T2 cheeses. In addition, the levels of this fraction in T3 cheese were statistically the same as those in T2 cheese but different in controls and T1 cheeses (Figure 1a). On the other hand, when the holding time increased (from 5 to 10 min), the levels of SN-pH 4.6 tended to increase for each level of pressure assayed. As for the SN-TCA, control cheese had a significantly ($P < 0.05$) lower level than all the HHP-treated cheeses, which, in turn, showed significant differences ($P < 0.05$) between

cheeses pressurized at 100 MPa and those pressurized at 400 MPa, which had the highest level (Figure 1b).

Interestingly, the cheeses treated by HHP at 400 MPa reached similar values for both indexes of proteolysis as control cheeses, 30 d earlier (Figure 1). Control cheeses showed levels of SN-pH 4.6 of $13.08 \pm 0.87\%$ and SN-TCA of $10.53 \pm 0.65\%$ at the end of ripening (90 d), and similar values were obtained for T4 and for T3 and T4 cheeses, respectively, at 60 d of ripening.

Electrophoresis. The electrophoretic profiles of the pH 4.6-insoluble fraction of the cheeses of 2 replicate

cheese makings are shown in Figure 2. The other 2 replicates were similar, and they are not shown. At the beginning of ripening (d 1 after HHP treatment), all electrophoretic profiles were similar. As was observed for the SN fractions, the profiles of the control and 100-MPa HHP-treated cheeses showed lower levels of proteolysis than the 400-MPa HHP-treated cheeses at 45 d. As visually observed, the intensity of the band of α_{S1} -I f(24–199) at 45 d was higher in the cheeses HHP treated at 400 MPa. At the end of ripening, the intensity of this band in all cheeses was similar.

Table 2. Values (means \pm SE) of the 4 replicas of cheeses for gross composition (%), pH, thermophilic and mesophilic lactobacilli, plasmin activity, coagulant activity, Asp, and total free AA

Item and ripening time (d)	HHP treatment ¹					SE
	Control	T1	T2	T3	T4	
Protein in DM (%)						0.92
1	50.53	50.82	51.19	50.93	51.65	
45	50.99	50.87	51.04	51.55	50.18	
90	50.02	49.35	49.68	50.26	49.68	
Fat in DM (%)						1.35
1	44.54	43.55	43.33	43.24	43.26	
45	45.52	43.26	44.44	44.67	44.70	
90	41.95	42.79	42.15	43.30	42.56	
Moisture (%)						0.27
1	37.80	38.13	38.49	38.26	38.44	
45	34.14	34.13	34.20	34.53	34.56	
90	33.57	33.38	33.54	34.17	33.91	
pH						0.03
1	5.18	5.19	5.19	5.23	5.26	
45	5.23	5.28	5.29	5.29	5.29	
90	5.27	5.28	5.30	5.29	5.30	
Thermophilic lactobacilli (log ₁₀ cfu/g)						0.32
1	8.72	8.58	8.89	7.69	6.89	
45	3.41	3.32	3.60	3.12	3.35	
90	0.80	0.83	0.83	0.85	0.84	
Mesophilic lactobacilli (log ₁₀ cfu/g)						0.16
1	8.04	8.13	8.67	7.82	7.65	
45	6.79	6.78	6.66	7.20	7.30	
90	6.29	6.69	6.72	7.01	6.91	
Plasmin activity ²						0.60
1	6.04	7.80	8.37	9.63	18.83	
45	3.96	5.75	5.64	8.24	10.11	
90	3.73	5.28	5.88	10.32	12.91	
Coagulant activity ³						24.04
1	158.85	164.59	157.87	165.90	182.28	
45	196.71	213.28	253.30	259.84	292.81	
90	198.86	216.80	190.22	203.03	202.46	
Asp ⁴						16.4
1	4.7	4.6	4.6	6.9	12.9	
45	52.6	82.7	71.4	121.3	113.0	
90	188.1	214.6	212.0	242.8	253.1	
Total free AA ⁴						430.9
1	247.4	247.5	257.2	329.1	590.7	
45	1,400.6	2,402.5	2,131.9	2,764.1	2,642.2	
90	2,888.4	3,263.5	3,284.1	3,539.1	3,572.9	

¹HHP = high hydrostatic pressure. Pressure and holding time for each treatment: T1 = 100 MPa, 5 min; T2 = 100 MPa, 10 min; T3 = 400 MPa, 5 min; T4 = 400 MPa, 10 min.

²Nanomoles of 7-amido-4-methyl-coumarin released per gram of DM.

³Nanomoles of product released per hour and per gram of DM.

⁴Milligrams of free AA per 100 g of cheese.

Regarding the breakdown products of β -CN, higher intensity of the γ -CN fractions was found in cheeses HHP treated at 400 MPa at 45 d of ripening.

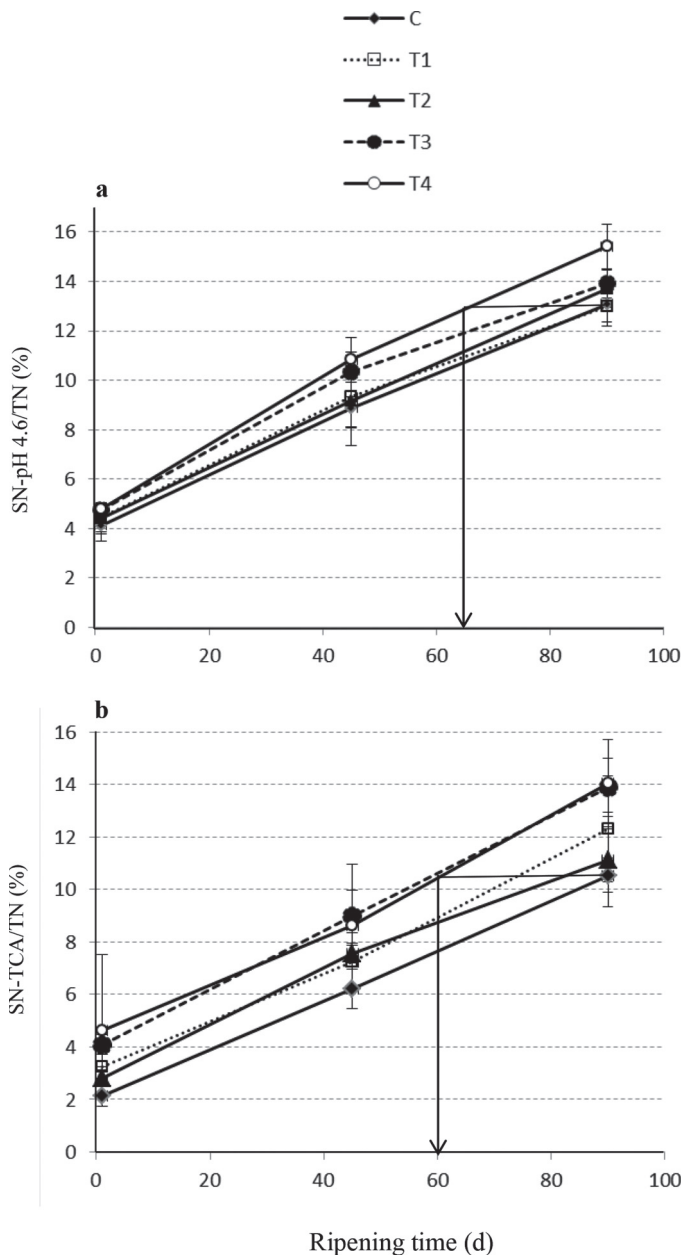


Figure 1. Soluble nitrogen (SN) (a) at pH 4.6 (SN-pH 4.6) and (b) in 12% (wt/vol) trichloroacetic acid (SN-TCA) expressed as the percentage of total N (TN) during ripening. For each ripening time, data points represent the mean \pm SE of 4 replicates. Arrows indicate the time at which the samples of cheeses treated with high hydrostatic pressure reached the same values of SN fractions as fully ripened control (C) cheeses. Pressure and holding time for each treatment: T1 = 100 MPa, 5 min; T2 = 100 MPa, 10 min; T3 = 400 MPa, 5 min; T4 = 400 MPa, 10 min.

Peptide Profiles in Cheeses by RP-HPLC.

Peptide profiles of all cheeses changed during ripening, with certain peak areas diminishing, whereas others increased (data not shown). The most evident change was the increase of peaks at the beginning of the chromatogram, which correspond to aromatic FAA—visible by UV detection in the condition of the assay—and hydrophilic peptides. The increase in the proportion of these compounds is the result of the usual evolution of proteolysis during ripening. Conversely, certain variations were found by visual comparison between peptide profiles of cheeses with different HHP treatments.

After variable reduction by fuzzy approach, peptide profiles were sorted into 19 classes of retention time, which were identified correlatively from c1 to c19. The areas of these classes were considered as input variables for principal components analysis, with standardization to a mean of zero and their original variances (covariance matrix). Two principal components (PC) with an eigenvalue higher than the mean of eigenvalues were extracted, which explained 96.2% of the total variance. Scores and loading plots for PC1 versus PC2 are presented in Figures 3a and 3b.

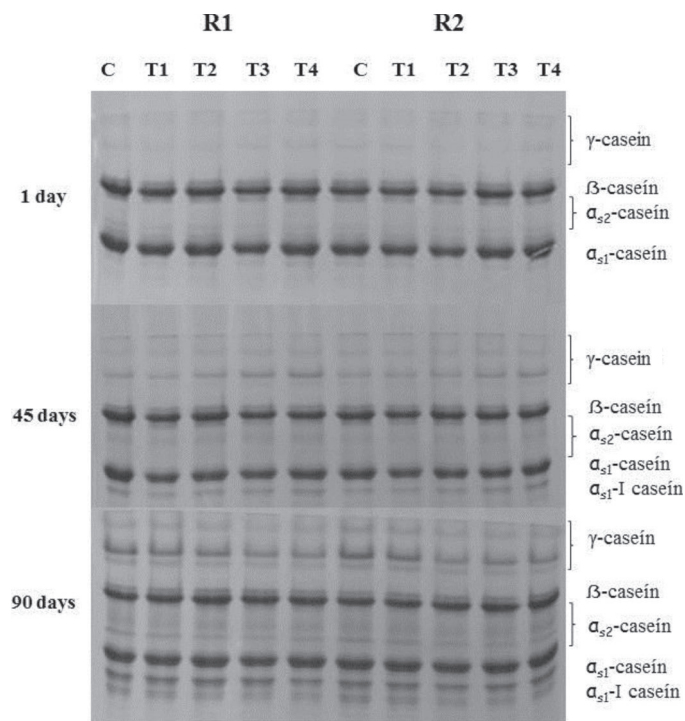


Figure 2. Electrophoretic profiles of insoluble cheese fractions at pH 4.6 during ripening. R1 = replicate 1, R2 = replicate 2; C = control. Pressure and holding time for each treatment: T1 = 100 MPa, 5 min; T2 = 100 MPa, 10 min; T3 = 400 MPa, 5 min; T4 = 400 MPa, 10 min.

In the score plot, cheese samples are distributed along the first PC according to their ripening time. In this way, 1-d-old cheeses had negative scores on PC1, 45-d-old samples were near to zero, and 90-d-old samples had positive scores. These results showed

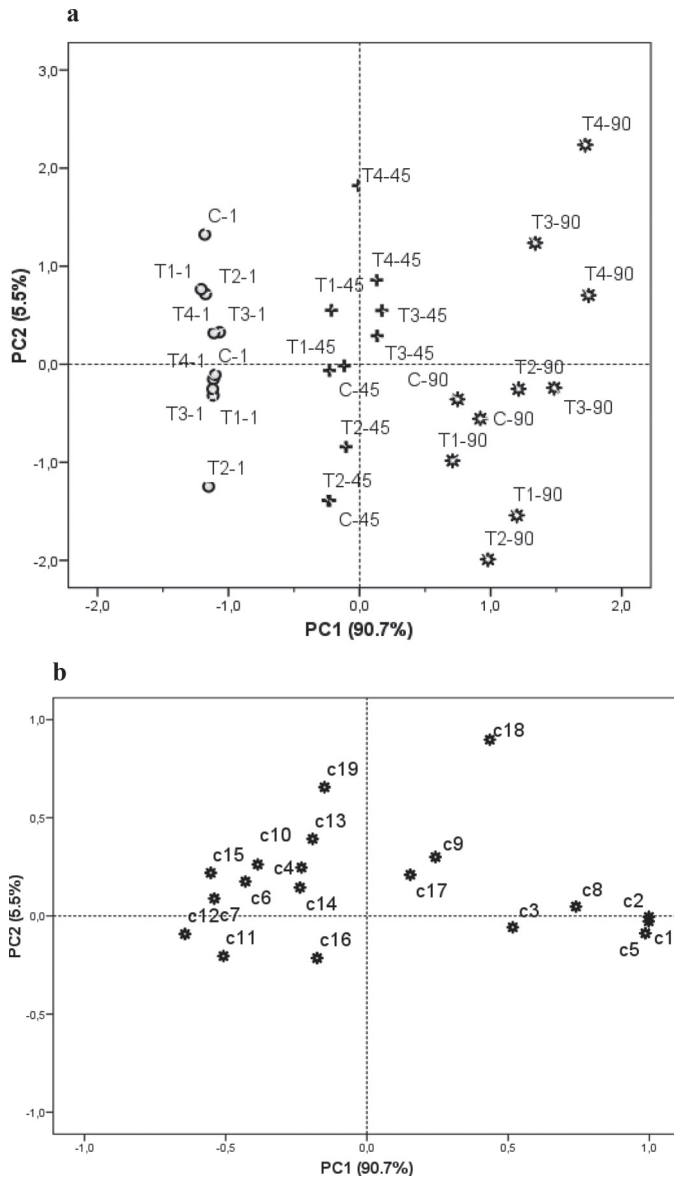


Figure 3. Principal component (PC) analysis of chromatographic profiles. (a) Score plot of samples at 1 (○), 45 (+), and 90 (*) d of ripening on PC1 and PC2. Pressure and holding time for each treatment: T1 = 100 MPa, 5 min; T2 = 100 MPa, 10 min; T3 = 400 MPa, 5 min; T4 = 400 MPa, 10 min. (b) Loadings plots for PC1 versus PC2. Classes of retention time (c) were as follows: c1 = 4.30 min, c2 = 8.45 min, c3 = 12.60 min, c4 = 16.75 min, c5 = 20.90 min, c6 = 25.05 min, c7 = 29.20 min, c8 = 33.35 min, c9 = 37.50 min, c10 = 41.65 min, c11 = 45.80 min, c12 = 49.95 min, c13 = 54.10 min, c14 = 58.25 min, c15 = 62.4 min, c16 = 66.55 min, c17 = 70.70 min, c18 = 74.85 min, and c19 = 79.00.

that the principal source of variation between samples was the ripening time, as PC1 explained most of the total variance (90.7%). Conversely, although samples of control and treated cheeses had similar scores on PC1 at 1 d of ripening, a separation between them was observed at 45 and 90 d. Overall, cheeses subjected to the most severe treatment (T3 and T4) had higher scores on the first PC than the other cheeses with the same ripening time. Because PC1 is related to ripening time, the higher values of PC1 for T3 and T4 cheeses suggest an acceleration of proteolysis. Variables c1, c2, c3, c5, and c8, which correspond with initial classes with retention time lower than 40 min (FAA and hydrophilic peptides), and also c18, whose retention time was 74.85 min, had higher loading on PC1, and so their increase is related to the increase of ripening time (Figure 3).

Conversely, HHP treatment had a clear influence on the sample scores on PC2, which explained 5.5% of variance. Overall, T3 and T4 samples, both at 45 and 90 d of ripening, showed higher scores on PC2 than the rest of samples at the same ripening time. Variables c18, c19, c13, and c9, which correspond with peaks that eluted in the middle and end of the chromatograms (hydrophobic peptides), showed the higher loading values on PC2 and thus are related to the applied treatment. These results reveal that HHP treatment at 400 MPa, besides its effect on acceleration of ripening by increasing peptide levels that typically increase during ripening in this type of cheese, produces a differentiation in this process. This treatment also resulted in a preferential increase of characteristic peaks (Figure 3).

FAA Analysis. Results of statistical analysis and concentrations of aspartic and total FAA of all experiments are shown in Tables 1 and 2. Total FAA concentration increased with ripening time and showed the highest levels at 90 d. This was also true for most individual FAA, except for Ala, His, and Trp, which increased to 45 d and then remained constant up to 90 d of ripening (data not shown). The HHP treatments of cheeses did not significantly affect the FAA profile, except for Asp, the levels of which significantly increased with the intensity of the HHP treatment. As observed for the SN fractions, samples of treatments T3 and T4 reached at 50 d of ripening levels of total FAA comparable to those of the control at 90 d.

DISCUSSION

In the present work, we studied the effect of HHP treatment on the activity of plasmin and residual coagulant, as well as on the proteolysis and peptidolysis of Reggianito cheese. In addition, the effect of HHP

treatment on gross composition, pH, and microbial counts was investigated.

The composition of the cheeses was not significantly affected by HHP treatment. Moisture content was comparable for all samples. Similar results were reported by Rynne et al. (2008) in Cheddar cheeses, but other works reported higher moisture in several varieties of cheese at the end of ripening when they were treated at 300 MPa or higher pressures (Saldo et al., 2000; Juan et al., 2008). In these cases, the higher moisture content at the end of ripening was attributed to changes in the casein network and higher water holding capacity in HHP-treated cheeses. In effect, the authors postulate that the increase in the levels of small peptides, which was verified in treated cheeses, led to a reduction in the loss of water that normally occurs during the ripening time. In our work, although T3 and T4 cheeses had higher moisture content on d 90 of ripening, the difference was not statistically significant, probably because the cheeses were vacuum packaged from d 12 onward, which prevented a subsequent drying of cheeses.

Regarding fat and protein, our results confirmed most previous research, which reported no changes attributable to HHP treatment (Saldo et al., 2002; Juan et al., 2008; Rynne et al., 2008; Moschopoulou et al., 2010). On the contrary, whereas several authors found differences in pH between standard and HHP-treated cheeses (Saldo et al., 2002; Rynne et al., 2008; Ozturk et al., 2013a), in our work the pH of cheeses was not affected by HHP treatment, though it increased significantly during ripening. The increase in pH during ripening of hard cheeses has been reported previously and is probably due to a combination of the proteolysis process and solubilization of insoluble calcium phosphate that occur during ripening (Lawrence et al., 1987).

The effect of HHP on the cheese microbiota was detected on d 1 after HHP treatment: thermophilic lactobacilli counts were lower in cheeses treated at 400 MPa than in the other cheeses, both controls and those treated at 100 MPa. Similar results were observed in Cheddar cheeses after HHP treatment at 400 MPa (Wick et al., 2004; Rynne et al., 2008; Ozturk et al., 2013b), yeast-ripened cheeses (Voigt et al., 2010; Calzada et al., 2014), goat cheese (Saldo et al., 2000), and sheep cheeses (Moschopoulou et al., 2010). Unlike the starter used in this work, consisting of *Lactobacillus helveticus* strains, in Cheddar, goat, and sheep cheeses the starter was composed predominantly of strains of *Lactococcus lactis*. The effect of HHP treatment on microorganisms depends on the process conditions (compression rate, pressure level, holding time, and temperature), food characteristics (composition, pH, and water activity), and the microorganism (genus, species, and strain)

(Cheftel, 1995; Huppertz et al., 2006). In real food systems, other factors play a role, because bacteria can recover after HHP treatment, which is often attributed to a protective effect by the food matrix (Wick et al., 2004). In fact, in Cheddar cheese with different salt concentrations (Ozturk et al., 2013b), it was observed that the effect of HHP on bacterial numbers increased with salt reduction, confirming the baroprotector effect of salt present in cheeses on bacteria. The decrease in microbial counts due to HHP treatment may indicate inhibition of growth of microorganisms, damage of the cell wall, or autolysis (Malone et al., 2002; O'Reilly et al., 2002). When autolysis of microorganisms occurs, the release of intracellular peptidases has a positive effect on cheese ripening acceleration (Wick et al., 2004). Juan et al. (2007) verified autolysis of a starter of *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* due to HHP treatment, which was confirmed by increased activity of the cytoplasmic enzyme lactate dehydrogenase in the HHP-treated cheeses. Malone et al. (2002) showed that treatment with HHP caused inactivation, physical damage, and autolysis of the same *Lactococcus lactis* strain and that extent of lysis was dependent on the strain and the pressure level used. Holding time had no effect on bacterial counts, which was in agreement with the results of Ozturk et al. (2013a).

As ripening progressed, thermophilic microbial counts decreased in all cheeses, and differences between HHP-treated and control cheeses were no longer detected. A drastic reduction was found at 45 d from 10^8 to 10^7 cfu/g on cheeses 1 d after HHP treatment to 10^3 cfu/g, and at the end of ripening, thermophilic lactobacilli was undetectable. Thermophilic lactobacilli loads at the beginning and end of ripening were comparable to those reported for Reggiano Argentine cheese by other authors (Candioti et al., 2002; Ceruti et al., 2014). Parmigiano Reggiano and Grana Padano cheeses, which are made with natural starters composed primarily of *Lactobacillus helveticus*, show similar trends as shown in this study, reducing levels to undetectable values at the end of ripening (Giraffa et al., 1997; Coppola et al., 2000), which is largely due to the autolytic activity of most strains of *Lactobacillus helveticus* (Sadat-Mekmene et al., 2013; Gatti et al., 2014).

In this work, markers of *Lactobacillus helveticus* lysis, such as intracellular enzymes, were not assessed. However, a detailed description of the peptidolysis, which also constitutes an index of the activity of intracellular peptidases of lactic acid bacteria in cheese, was undertaken. In this sense, the early decrease in thermophilic lactobacilli population may have contributed to accelerated peptidolysis and was, in fact, consistent with

an increase of global proteolysis observed in cheeses treated to 400 MPa, as discussed below.

Overall, during cheese ripening, the nonstarter lactic acid bacteria begins to prevail, while the number of lactic acid bacteria from the starter decreases (Sousa et al., 2001; Gatti et al., 2014). This trend was also verified in the present work. The counts of mesophilic lactobacilli at the end of ripening, which are mainly nonstarter lactic acid bacteria, were similar to those found in previous research on Reggiano Argentinio cheese (Ceruti et al., 2014). The decrease in the number of mesophilic lactobacilli due to HHP treatment at 400 MPa at the beginning of ripening was also found in other varieties of cheese when similar pressures were applied at 1 d after cheesemaking, as in our study (Juan et al., 2007; Rynne et al., 2008; Moschopoulou et al., 2010).

Plasmin and residual coagulant are proteolytic enzymes that are normally active in the cheese and involved in primary proteolysis. Previous studies have reported that the application of HHP treatment at the beginning of ripening can affect cheese ripening through modulation of enzymatic reactions (Messens et al., 1999; O'Reilly et al., 2000). In this work, plasmin activity in cheeses treated at 400 MPa during 10 min was increased. This activation was not previously reported in cheeses. In Cheddar cheese, when a similar HHP treatment was applied, the activity of this enzyme was not affected by HHP treatment (O'Reilly et al., 2002; Huppertz et al., 2004; Rynne et al., 2008). Evidence exists that the application of low to moderate hydrostatic pressures (100 to 400 MPa) can increase the activity of proteolytic enzymes (Eisenmenger and Reyes-De-Corcuera, 2009), whereas higher pressures (>400 MPa) can inactivate them (Huppertz et al., 2004). The range of pressures in which the HHP treatment can switch from positive to negative effects on the activity of an enzyme probably depends on the characteristics of the enzyme and the food matrix but also on other components of interest in the enzyme system. Plasmin has a complex system of precursors and inhibitors in the matrix milk, where the inactive precursor plasminogen is also included. Whereas some of these components are lost in the whey during cheesemaking, others are retained in the curd in a proportion depending on the technology applied for cheesemaking. In addition, the activation of plasminogen to different degrees is also dependent on the technology applied (McSweeney, 2004; Ismail and Nielsen, 2010). Therefore, it is difficult to compare the effect of a given HHP treatment among different varieties of cheese. As for the residual coagulant activity, it was not affected by HHP treatment, which agrees with data reported in previous studies (Messens et al., 1999; O'Reilly et al., 2002; Huppertz et al., 2004; Rynne et al., 2008).

α_{S1} -Casein is the most susceptible protein in the cheese matrix to the activity of coagulant enzyme during cheese ripening, and the primary site of attack is Phe₂₃-Phe₂₄ to produce peptides α_{S1} f(1-23) and α_{S1} -I f(24-199; Carles and Ribadeau-Dumas, 1985). As for plasmin, it is known that the preferential substrates are α_{S2} -CN and β -CN, with the latter giving the C-terminal fragments known as γ 1-CN, γ 2-CN, and γ 3-CN (Rampilli and Raja, 1998). An increase in the concentration of γ -CN is considered an index of increased plasmin activity (Grufferty and Fox, 1988). In this work, all of the indexes of proteolysis assessed showed the same overall trend: this primary biochemical transformation of cheese ripening was influenced by HHP treatment. The effect was most evident when treatment at 400 MPa was applied, and it was verified for all the SN fractions and electrophoresis, with a higher intensity in α_{S1} -I fractions and γ -CN fractions, especially after 45 d of ripening. The greater intensity of γ -CN was consistent with an increased activity of plasmin in cheeses HHP treated at 400 MPa. Messens et al. (1998) also showed an increase in β -CN hydrolysis, even when they found no changes in plasmin activity in Gouda cheeses treated at 300 MPa. These authors attributed the differences to a conformational change in the structure of the casein as a result of the pressure, leading to greater exposure of susceptible bonds from β -CN that became readily cleavable by plasmin. Saldo et al. (2002) also found a higher concentration of the products of β -CN hydrolysis by plasmin in goat cheeses treated 5 min at 400 MPa; however, no difference was found in plasmin activity compared with control cheese. In this case, the authors explained this fact by the higher pH of the cheeses treated by HHP, which is closest of the optimum pH for this enzyme.

For the α_{S1} -I fraction, the increase verified in our work did not correlate with increased residual coagulant activity because coagulant activity did not significantly differ between treatments. A similar trend was reported by Rynne et al. (2008).

Changes in cheese primary proteolysis attributable to HHP treatment were observed previously for different cheese varieties. Rynne et al. (2008) found an increased hydrolysis of α_{S1} -CN and higher levels of SN-pH 4.6 in Cheddar cheese treated at 400 MPa for 10 min. Accelerated primary proteolysis was also reported for cheeses treated at ~400 MPa for goat cheese (Saldo et al., 2000), ewe milk cheese (Garde et al., 2007; Juan et al., 2007), and Gouda cheese (Messens et al., 2001). Contrary results were reported by Sheehan et al. (2005) in Mozzarella cheese that was similarly pressurized, which exhibited no changes in primary proteolysis. Ozturk et al. (2013a) found similar behavior in reduced-fat Cheddar cheese, in which the pressure and holding

time varied between 48 and 402 MPa and 2.5 and 19.5 min, respectively. However, in this study, HHP was applied a week after cheesemaking, which suggested that cheese age is a relevant factor. In Edam cheese treated at pressures of 200 and 400 MPa at 4, 6, or 8 wk after cheesemaking, an initial decrease in the SN-pH 4.6 fraction after HHP treatment with respect to untreated control cheese was observed. However, at the end of ripening, SN levels of all cheeses were similar (Iwanczak and Wisniewska, 2005). In our study, the holding time had only an effect on SN fraction at pH 4.6 and plasmin activity; cheese treated for 10 min showed higher values than those treated for 5 min, at both levels of pressure assayed. In other works in which similar treatments were used, different holding times had no effects on primary proteolysis (Messens et al., 1999; Ozturk et al., 2013a). These results show that the physicochemical characteristics and the specific environmental conditions of different cheeses studied, as well as the time after cheesemaking in which the HHP treatment is applied, all have a strong effect on the effect of HHP treatment on cheese proteolysis.

The peptide profiles of our samples treated at the highest pressure also differed from the rest, which showed a higher secondary proteolysis in T3 and T4 cheeses. We also found an overall higher amount, although not significant, of total FAA when the pressure increased, but individual concentrations of FAA showed no change, with the exception of aspartic acid, the concentration of which was significantly higher in the cheeses treated at 400 MPa. This absent or notably small effect of HHP treatment on the levels of FAA was also observed by other authors in Cheddar cheese (O'Reilly et al., 2002; Rynne et al., 2008), Mozzarella (Sheehan et al., 2005), and Gouda (Messens et al., 1999). In ewe cheese, no effect of treatment in the level of total FAA was found for cheeses treated at pressures greater than 400 MPa, but the cheese treated at lower pressure (200 to 300 MPa) had higher levels of total FAA than the controls (Juan et al., 2007). It is important to consider that the content of FAA in a cheese is the result of the balance between their production from peptides and their catabolism. So, the effect of HHP treatment on the FAA levels could be masked if the activity of both peptidases and enzymes involved in the catabolism of FAA are increased.

CONCLUSIONS

The HHP treatment of Reggianito cheese had no influence on gross composition or pH at any time of ripening studied, and treatment at the highest pressure decreased starter counts and increased plasmin activ-

ity. Consistently with these findings, proteolysis and peptidolysis were accelerated in the 400 MPa-treated cheeses, which were as proteolysed at 60 d of ripening as were the control unpressurized 90-d-old cheeses. Cheeses treated at 100 MPa did not exhibit differences from unpressurized samples. According to the results obtained in our study, HHP treatment at 400 MPa is promising to accelerate cheese ripening of hard cooked cheese. Because the pilot plant in which the study was conducted reproduces in small scale the processing conditions of a plant on a commercial scale, these results should be validated by means of experiences in large scale.

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