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Effect of High-Pressure Processing on Proteolysis, Texture and Sensorial Attributes of Raw Ewe's Cheeses Throughout Storage

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Abstract: *Serra da Estrela* cheese, with a Protected Denomination of Origin (PDO), is one of the most appreciated traditional raw milk Portuguese cheeses, and it is well known for its unique flavor and texture, which are derived from the use of raw ewe's milk and its production process. In this work, 45-day-old ripened *Serra da Estrela* cheeses were processed by high-pressure processing (HPP) at 600 MPa/6 min (P1), 450 MPa/6 min (P2) and 450 MPa/9 min (P3) to study the effect of HPP initially and during 15 months of storage at 4 °C. The proteolysis indexes were, in general, lower in the HPP-treated cheeses than in the control cheeses. The P1 cheeses kept their ripening extension index throughout the 15 months of storage close to that of non-processed cheese at month 0. Progression of the ripening depth and free amino acids indexes was also slowed down by HPP. HPP had no immediate effect on the cheese texture parameters, and minor changes were found up to 3 months of storage; moreover, the P2 cheeses maintained their hardness and consistency levels during the 15-month storage period at values close to those of the control cheeses at month 0. Sensory evaluation by trained panelists showed that the P2 cheeses were softer than the control cheeses; furthermore, for the P3 cheeses, there were no observed treatment effects on the sensory attributes evaluated at the end of storage. Overall, the results uphold the potential of HPP in rendering *Serra da Estrela* cheese proteolysis levels similar to those of control cheese at 45 days of ripening with minor effects on texture.

Keywords: high-pressure technology; Serra da Estrela cheese; raw ewe's milk; proteolytic indexes; aminopeptidase enzyme; texture

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1. Introduction

Raw ewe's milk *Serra da Estrela* cheese, with Protected Denomination of Origin (PDO) certification in the European Union, owes its unique characteristics mainly to the milk's origin and the traditional manufacturing process. *Serra da Estrela* cheese is made exclusively with raw milk from Bordaleira and/or Churra Mondegueira sheep breeds, salt and a crude plant rennet extract (dried flowers of *Cynara cardunculus* L.), and it is commercialized only after 30 days of mandatory ripening [1,2]. These conditions result in a cheese with distinctive organoleptic characteristics, with a closed, moderately buttery, deformable when cutting, well connected, creamy and unctuous texture, with few or no eyes and a smooth, clean and slightly acidic *bouquet* [3]. These organoleptic features are largely associated with proteolysis, the most important biochemical and complex process in cheese ripening.

Proteolysis in cheese can be evaluated via proteolytic indexes, in particular by the ripening extension index (ratio of water-soluble nitrogen to total nitrogen—WSN/TN ratio). For *Serra da Estrela* cheese, this index was found to increase from 9.5–11%, just after manufacturing to 23–59% after 35–180 days of ripening [4–7]. These high values were associated with the role that the vegetable rennet plays in cheese proteolysis [8], causing more extensive proteolysis, which is associated with a more homogeneous cheese structure and increased creaminess and softness [9]. It is known that cardoon flowers influence the texture of cheese [10].

Since *Serra da Estrela* cheese is a non-pasteurized product manufactured from raw milk, microbial safety is a pertinent issue. From a safety point of view, previous results have demonstrated the usefulness of HPP (400–600 MPa, 3–10 min) to render *Serra da Estrela* cheese microbiologically safer during a more extended shelf-life of up to 100 days under refrigeration [11]. The observed effect of HPP on cheese properties is dependent on the pressure intensity, the holding time under pressure and the cheese ripening stage at which HPP is applied.

The effect of HPP on proteolysis in whole cheeses manufactured with raw ewe's milk has been studied during ripening of La Serena cheese [12] and during storage of Casar [13] and Torta del Casar [9] cheeses and for small pieces (~20 g) of *Serra da Estrela* cheese [11]. La Serena cheese that was pressure-treated at 300 and 400 MPa (10 min, 10 °C) at 50 days of ripening showed no changes in proteolysis level after 10 days under conventional ripening conditions [12]. During storage of Casar cheese, casein degradation was significantly retarded in HPP-treated cheeses (600 MPa/5 min) [13]. Also, Delgado et al. (2015) [9] treated Torta del Casar cheese at 600 MPa/5 for 20 min at 60 days of ripening and demonstrated a reduction in proteolysis of casein fractions (WSN/TN ratio decreased) after 240 days of storage. In a previous study with small portions of *Serra da Estrela* cheese, pressure treatments caused no significant changes in WSN content up to 100 days of refrigerated storage [11].

According to the above-mentioned studies, HPP treatments applied at the end of the ripening period slowed down or maintained the casein hydrolysis state and nitrogen ratio during storage. Since proteolysis has a marked effect on texture and, thus, on *Serra da Estrela's* sensorial characteristics, this type of HPP treatment opens an interesting possibility to maintain the degree of cheese proteolysis during extended storage and, thus, to avoid undesired changes in texture. HPP can be used to process Serra da Estrela cheese in conditions that assure microbial safety without influencing cheese lipid profiles [14].

Thus, in this work, the effects of HPP (450 MPa for 6 or 9 min or 600 MPa for 6 min) applied to whole *Serra da Estrela* cheeses after 45 days of ripening on the evolution of cheese proteolysis, texture and sensorial properties over 15 months of refrigerated storage were studied.

2. Materials and Methods

2.1. Cheese Manufacturing and High-Pressure Processing

Two batches of *Serra da Estrela* cheese were manufactured using 150 L of unpasteurized ewe's milk according to the required PDO guidelines [1,2]. Due to the limited production capacity, one batch was manufactured in the morning (batch A) and the other in the afternoon (batch B) on the same day. The produced fifty-six Serra da Estrela cheeses (~0.5 kg) were ripened for 45 days according to the PDO procedures [1]. During the first fifteen days, the cheeses were ripened in a chamber at 9 °C and 95% relative humidity; thereafter, the cheeses were transferred to a second ripening chamber at 11 °C and 75%

relative humidity. The ripened cheeses were then pressurized for 6 min at 600 MPa (P1) or at 450 MPa for 6 (P2) and 9 min (P3), with unpressurized cheeses being kept as controls (Ch_C). The HPP treatments were performed in a 55 L capacity industrial-scale high-pressure device (model 55, Hiperbaric, Burgos, Spain). After HPP, the cheeses were kept for 15 months under refrigerated storage (4 °C). Four cheeses from each batch were taken at each sampling point—0, 1.5, 3, 6 and 15 months of storage—and analyzed. Four more cheeses were taken at each sensorial analysis sampling point—0, 1.5, 6 and 15 months of storage. At each sampling point, twelve samples of homogenized cheese core, 75 g each, were frozen at −80 °C until further chemical analysis.

2.2. Proteolytic Indexes

Proteolysis was monitored during cheese storage by measuring the amount of water-soluble nitrogen (WSN), 12% (w/v) trichloroacetic-acid-soluble nitrogen (TCA) and 5% (w/v) phosphotungstic-acid-soluble nitrogen (PTA) in the cheese extracts via the micro-Kjeldahl method [15,16] using a Kjeltec system with a 2012 digester and a 1002 distilling unit (Tecator, Hoganas, Sweden). The cheese extracts were obtained according to the method of Macedo and Malcata (1997) [4]. The analyses were run in duplicate per cheese (four analyses). The contents of WSN, 12% TCA and 5% PTA are expressed as the per unit mass of the total nitrogen content (TN). The following ripening indexes were calculated: ripening extension index (WSN/TN), ripening depth index (TCA/TN) and free amino acid index (PTA/TN).

2.3. Aminopeptidase Activity

Aminopeptidase activity was measured in triplicate on an extract obtained by homogenizing 10 g of cheese with 20 mL of 10 mM sodium phosphate buffer, pH 7, at room temperature for 4 min in a Stomacher 80 (Biomaster; Seward Laboratory Systems Inc., Davie, FL, USA), followed by centrifugation (10,000× g, 15 min, 4 ± 1 °C) and filtering through Fiorinni 112A filters. Lysine p-nitroanilide (Lys-p-NA) and leucine p-nitroanilide (Leu-p-NA) were used as substrates at 1 mM in 50 mmol TRIS-HCl buffer, pH 7.0. The reaction mixture consisted of a 275 µL substrate solution and a 25 µL enzyme solution (the blank consisted of the same mixture without substrate in TRIS-HCl buffer). Assays were carried out at 30 °C using a microplate spectrophotometer (Multiskan Go, Thermo Scientific, Thermo Fisher Scientific Inc., Waltham, MA, USA) and a Nunc UV plate with 96 wells. The absorbance of the released p-nitroaniline was read at 410 nm in 2 min intervals. Aminopeptidase activities are expressed in nmol of released p-nitroaniline per minute per g of cheese and are presented as mean ± STD (n = 6) using triplicate determinations in two cheese-making experiments.

2.4. Instrumental Texture Profile Analysis (TPA)

Each cheese from each batch (batches A and B) was kept at room temperature (18–22 °C) for 2 h before analysis. Random cylinders of cheese (18 mm diameter) were taken with a cork borer inserted vertically through the cheeses from their top surface, crossing from side to side, being cut 3 mm per side, corresponding to the cheese rind. For the analysis of the texture, a TA-Hdi texturometer from Stable Micro system (Godalming, UK) was used, which was connected to a 2 mm diameter probe. Each test was conducted as 2 sequential penetration events with a 10 mm penetration at a rate of 0.80 mm/s, separated by a rest period of 10 s. The tests generated a force–time curve, from which the hardness (N), consistency (N/s), adhesiveness (N/s), cohesiveness, elasticity, gumminess (N), chewiness (N) and Young's modulus were calculated. All analyses were performed in sextuplicate per cheese.

2.5. Sensory Evaluation

Sensory analyses were carried out mid-morning, with sensory sessions taking place at an ISO 8589:2007 [17]-compliant sensory evaluation laboratory at Universidade Católica Portuguesa, equipped with white fluorescent lighting (6500 K). The analyses were carried out at room temperature (18–22 °C).

A panel of ten trained panelists was used. In each testing day, one cheese per sample was removed from refrigeration about 1 h prior to evaluation and kept at room temperature. The rind and the outermost layer (0.7 cm thickness) were removed, and the cheese paste was then cut into slices. The cheese slices were individually presented to the panelists in Petri dishes coded with three-digit random numbers (for hidden control, HPP P1, P2 and P3 cheese slices). Descriptions of the defects that were eventually found were also recorded. Mineral water and Granny Smith apple slices were provided to the panel members to cleanse their palates between samples. The panel sessions were held mid-morning. The order of presentation of the samples was randomized across panelists. An online Qualtrics questionnaire in Portuguese was used in the sensory sessions. A quantitative descriptive test was applied at a scale ranging from −10 to 10, with a value of 0 being equal to the control/reference. The attributes evaluated by the panelists were as follows: appearance (color, from much lighter to much darker, and consistency, from much more fluid to much firmer) odor (lactic, acid, animal/stable and short-chain fatty acids/vomit, from much less intense to much more intense), texture (consistency, from much softer to much harder, and friability, from much less friable to much more friable), taste (salty, acid and bitter, from much less intense to much more intense) and aftertaste (much less intense).

2.6. Statistical Analysis

Experimental data were analyzed by analysis of variance (ANOVA) to determine the main effects and interactions of the different processing conditions (three HPP treatments and the control—Ch_C) and storage on all the variables tested. Bonferroni's significant difference test was applied to compare the mean values of the parameters, with significance assigned at a p-value < 0.05. Sensory data were analyzed by Student's paired t-test, comparing the results for each HPP-treated cheese with the Ch_C (blind control sample), with the significance assigned at a p-value < 0.05. When the distribution of the differences between the control and treated cheeses failed to follow a normal distribution, Wilcoxon's non-parametric test was applied. The SPSS software version 24.0 was used for the statistical analysis.

3. Results and Discussion

3.1. Effect of HPP on Proteolytic Indexes

The assessment of the extent of proteolysis in cheese is of interest as an index of cheese maturity and quality. For each type of cheese, the ideal ripening time is established based on the achievement of the desired texture, aroma and flavor properties. The evolution of the three proteolytic indexes of the *Serra da Estrela* cheeses, immediately upon HPP and throughout 15 months of refrigerated storage, is shown in Figure 1. The ripening extension index (WSN/TN ratio) is typically used to follow the aging of cheese, with it being proportional to the proteolytic enzyme activity. It reflects the direct action of rennet, which is retained in the curd after manufacturing, on casein, and it consists of large to medium-sized peptides [18]. At the beginning of storage (0 months), the Ch_C cheeses were characterized by a WSN/TN ratio of 29 ± 2.0%. This index varies throughout ripening and all through the cheese-making season, with Macedo and Malcata (1997) [4,19] reporting WSN/TN values of between 27 and 36% for *Serra da Estrela* cheese at 35 days of ripening During the 15 months of refrigerated storage, this index increased steadily up to 1.5 months of storage, stabilized between 1.5 and 6 months of storage and then increased steadily again up to a final WSN/TN value of 45 ± 1.1% at 15 months of storage. Similar values, between 23–59%, were reported for this type of cheese within 35–180 days of ripening. In terms of the HPP-treated *Serra da Estrela* cheeses, different behaviors were observed compared to the control Ch_C cheese. At the beginning of storage (0 months), immediately after the HPP treatments with 6 min of holding time, independently of the pressure intensity (P1 and P2), there were no significant changes in the WSN/TN index ($p > 0.05$) compared to the Ch_C cheese. On the other hand, HPP treatment for a longer period of time (P3—450 MPa/9 min) caused a significant 17% increase in the corresponding WSN/TN index ($p < 0.05$) immediately after HPP, yet no further changes were observed over the 15 months storage of these P3 cheeses ($p > 0.05$). This observation may be related to the fact that a longer period of exposure (9 min) may have promoted further conformational changes in the casein matrix, making it more susceptible to proteolytic enzymes as compared to the 6 min exposure [20,21].

In general, a deceleration of the ripening extension index was observed for all the HPP-treated cheeses during the 15 months of storage, especially for those treated under higher pressure (P1, 600 MPa) ($p > 0.05$). This behavior was also observed by Delgado et al. (2015) [9] for HPP-treated Casar cheeses (600 MPa/20 min) at 60 days of ripening and after being stored for 240 days. Application of HPP at the end of ripening (42–60 days) also led to lower proteolysis levels for Ibores and La Serena cheeses [12,22]. Notably, the P1 cheeses kept their WSN/TN index stable during the whole storage period, with values ranging between 27–30%, i.e., values similar to those of the Ch_C cheese at 0 months (29% obtained at 45 days of ripening was considered to be the best condition to generate ideal sensorial properties). Such results indicate that this HPP treatment (600 MPa/6 min), when applied to 45-days-ripened *Serra da Estrela* cheese (0 months storage), may halt proteolysis during 15 months of storage, keeping cheese properties at ideal levels that are comparable to those of 45-day-ripened Ch_C cheeses. This observation is of considerable importance, since texture, aroma and flavor are influenced by proteolytic activity. The cheese matrix is in itself a network of casein particles, which disintegrates as proteolytic enzymes take action. A lower level of proteolysis leads to a more consistent cheese, whereas a higher level of proteolysis increases the softening and meltability of cheese [9]. Given the lower ripening extension index by 15 months of storage, the HPP P1 cheeses were in fact firmer and harder compared to Ch_C. This was confirmed by both instrumental (TPA profile) and sensorial analysis by the trained panel (see further details in Sections 3.3 and 3.4, respectively).

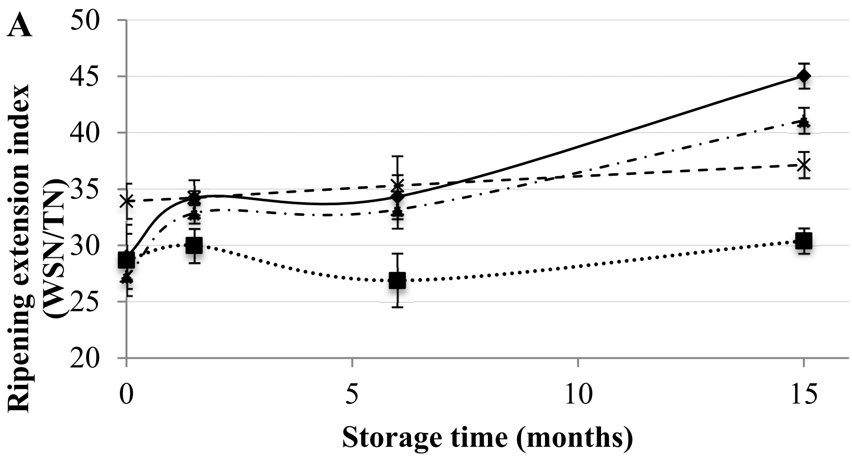

Figure 1. *Cont.*

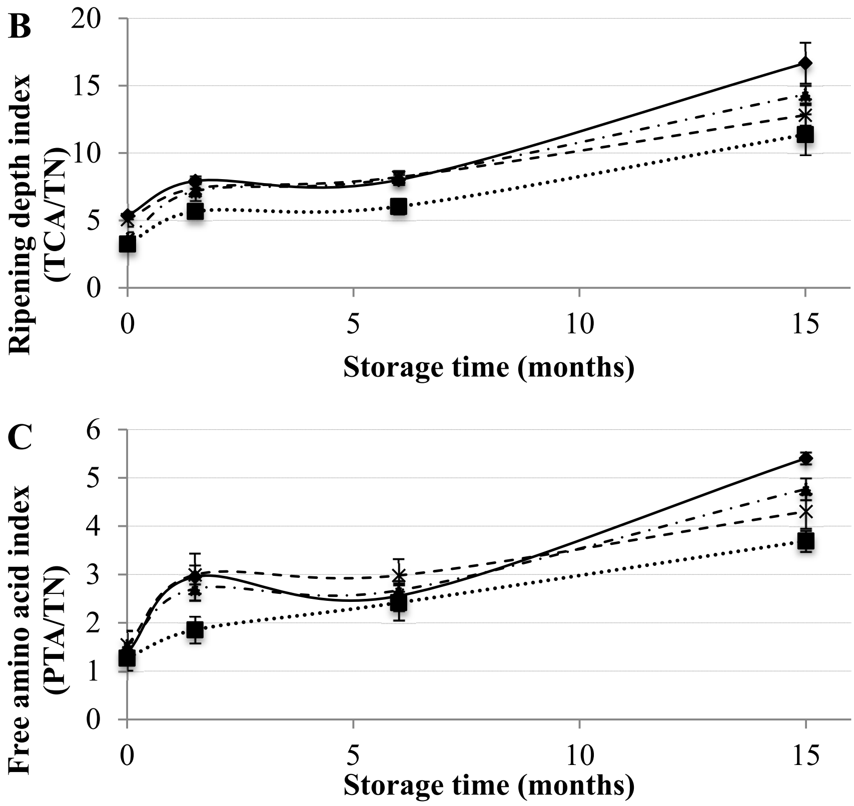

Figure 1. (**A**) Ripening extension index (WSN/TN), (**B**) ripening depth index (TCA/TN) and (**C**) free amino acid index (PTA/TN) of Serra da Estrela cheese at 45, 135, 225 and 500 days of manufacture for non-processed cheeses (◆, Ch_C) and HPP-treated cheeses (■ P1, ▲ P2 and **X** P3).

A significant progressive increase in the value of TCA/TN, understood as a ripening depth index ($p < 0.001$), was observed for all cheeses during the 15 months of refrigerated storage (Figure 1A); an intermediate plateau similar to that previously observed for WSN/TN was also registered. This proteolysis index expresses the presence of medium- and small-sized peptides (with a chain length between 2 and 20 amino acids residues) and free amino acids, which may result from the strong proteolytic action of *C. cardunculus* extract [6,23] but is mainly derived from peptidase activity from viable or lysed lactic acid bacteria [4,5,7] and/or from psychrotrophic bacteria [19]. In this study, the viable cell numbers of psychotropic bacteria were higher in the Ch_C and P2 and P3 cheeses compared to the P1 cheeses [24]. The obtained TCA/TN values are in agreement with other reported results; Macedo and Malcata (1997) [19] reported a TCA/TN value of 5.5–6.2% at 35 days of ripening for *Serra da Estrela* cheeses, Tavaria et al. (2003) [6] reported values of 7–16% at 35–180 days of ripening, while Reis and Malcata (2011) [7] reported values of 3.7% at 60 days of ripening. In general, there were no significant differences in the TCA/TN indexes between the Ch_C cheeses and the HPP P2 and P3 cheeses (450 MPa/6 and 9 min, respectively). On the other hand, significantly lower TCA/TN indexes were obtained for the HPP P1 cheeses treated at 600 MPa/6 min than for the Ch_C cheeses ($p < 0.001$) over the whole storage period. This observation may be consequence of a reduced microbial peptidase activity that could be linked with the reduction in microbial viable cell numbers caused by HPP, as observed in a previous work [24]. This effect was also observed in HPP-treated Casar cheeses (600 MPa/20 min) at 60 days of ripening and after being stored for 240 days, with Delgado et al. (2015) [9] suggested that HPP at 600 MPa led to a reduction in the production of medium to small-sized peptides. On the other hand, Delgado, González-Crespo, Cava and Ramírez (2012) [25] observed an increase in TCA/TN in Ibores cheese (raw goat milk) HPP-treated (400 and 600 MPa/7 min) at 50 days of ripening compared to control cheeses, possibly due to the intracellular release of proteinases/peptidases [25]. These results seem to demonstrate that high-pressure treatments may accelerate or decelerate cheese proteolysis based on the cheese type and the pressure conditions used [26]. The PTA/TN ratio, used as a free amino acid (FAA) index, is related to the final product of proteolysis, the FAA and very small peptides (containing less than six amino acid residues). Once again, the PTA/TN ratio significantly ($p < 0.05$) increased progressively during refrigerated storage for all the non-treated and HPP-treated cheeses (Figure 1C). In the case of the Ch_C cheeses, the values of PTA/TN increased almost 4-fold, going from 1.4 ± 0.2 at 0 months storage to 5.4 ± 0.1 at 15 months of storage. These values are within the range previously reported by Tavaria et al. (2003) [6], i.e., 3–12% at 60–180 days of ripening, and they are also in line with the increase from 0.56 to 2.6% during the first 60 days of ripening reported by Reis and Malcata (2011) [7] for the same type of cheese. This FAA index has been associated with the hypothesis that FAAs are released by means of peptidases synthesized by adventitious microorganisms in *Serra da Estrela* cheese [6]. In fact, the lowest value of PTA/TN determined for the HPP-treated P1 cheeses paralleled the higher reductions in viable cell numbers observed in these cheeses [24]. On the other hand, HPP-treated P2 and P3 cheeses, which revealed similar PTA/TN ratios compared to that of the Ch_C cheeses ($p > 0.05$), suffered a minor effect on microbial composition. Delgado et al. (2015) [9] also observed a similar FAA content between Casar cheeses HPP-treated (600 MPa/20 min) at 60 days and a respective control.

3.2. Effect of HPP on Aminopeptidase Activity

HPP is capable of inactivating microorganisms, but it can also affect enzyme activity due to their (in)activation or to changes in their substrates' conformation [21]. It is well established that aminopeptidase activity contributes significantly to proteolysis in cheese [27], and so its activity was quantified on both Lys-p-NA and Leu-p-NA substrates, and the results are listed in Table 1. The activity decreased during the refrigerated storage for both substrates and for all cheeses, with the exception of the P1 cheeses, which showed a significant increment in the activity on Leu-p-NA from 0 to 6 months of storage ($p < 0.001$). Aminopeptidase activity on Lys-p-NA was significantly higher ($p < 0.001$) in the P1 (17% higher) and P2 (+59%) cheeses at 0 months than in the Ch_C cheeses. A similar behavior was observed throughout the 15 months of storage, although no significant activity variation was observed during the last 9 months of storage, for all the cheeses. On the other hand, all the cheeses registered a similar activity for Leu-p-NA at 0 months ($p > 0.05$); however, during the 15 months of refrigerated storage, the associated aminopeptidase activity decreased significantly ($p < 0.05$) in the Ch_C, P2 and P3 cheeses. It is important to note that aminopeptidases contribute toward the ripening depth index, taking into account the amount of nitrogen in the TCA fraction per TN. The TCA fraction allows for the quantification of small peptides containing between 2 and 20 amino acid residues and free amino acids resulting from secondary proteolysis, which is brought about by the enzymes produced by the starter cultures and released thereby upon lysis [28]. Trujillo et al. (2000) [29] suggested that HPP treatment could release intracellular enzymes due to incremented cell membrane permeability and microbial cells lysis, favoring the release of intracellular material, including peptidases. In general, an inverse relationship was observed between the proteolytic indexes and aminopeptidase activity during storage; as the values of the proteolytic indexes increased, the aminopeptidase activity decreased. Thus, aminopeptidase activity is not an adequate indicator of proteolysis in *Serra da Estrela* cheese. Indeed, other proteolytic enzymes will have their activities increased during storage and/or HPP, and storage may increase the proteolytic susceptibility of proteins. For instance, changes in cheese casein conformation or casein aggregation were reported to occur during HPP [12]. A higher aminopeptidase activity in HPP-treated cheeses (400 MPa/10 min) was also observed for La Serena cheese treated at 50 days of ripening and analyzed 10 days later, even though a similar level of proteolysis was quantified [12]. Furthermore, Juan et al. (2007) [30] also observed this tendency in HPP-treated ewe's cheese during ripening; the authors tested two HPP treatments, i.e., 400 MPa and 500 MPa for 10 min each, and although some aminopeptidase inactivation was observed at the higher HPP treatment (500 MPa/10 min), both HPP-treated cheeses revealed similar WSN contents compared to a control.

Table 1. Aminopeptidase activity of non-processed (Ch_C) and HPP-treated Serra da Estrela cheeses (P1, P2, P3).

Property	Storage Time (Months)	Ch_C	P1 600 MPa/6′	P2 450 MPa/6′	P3 450 MPa/9′
Activity for Leu-*p*-Na *	0	11.0 ± 0.8 ^{a,A}	11.2 ± 0.5 ^{a,B}	10.4 ± 0.8 ^{a,A}	10.4 ± 0.8 ^{a,A}
	3	9.8 ± 0.6 ^{b,B}	14.2 ± 0.6 ^{a,A}	10.1 ± 0.8 ^{b,A}	10.6 ± 0.2 ^{b,A}
	6	7.9 ± 0.5 ^{c,C}	13.3 ± 0.6 ^{a,A}	8.2 ± 0.3 ^{c,B}	9.8 ± 0.4 ^{b,A}
	15	5.3 ± 0.8 ^{c,D}	10.2 ± 0.9 ^{a,B}	6.7 ± 0.4 ^{b,C}	6.6 ± 0.6 ^{b,B}
Activity for Lys-*p*-Na #	0	21.9 ± 2.5 ^{c,A}	28.0 ± 4.1 ^{b,A}	34.9 ± 3.7 ^{a,A}	20.8 ± 2.6 ^{c,A}
	3	7.2 ± 1.4 ^{c,B}	20.6 ± 1.3 ^{a,B}	13.7 ± 2.2 ^{b,B}	17.6 ± 4.0 ^{a,A,B}
	6	7.7 ± 0.3 ^{d,B}	19.7 ± 2.1 ^{a,B,C}	13.5 ± 0.7 ^{c,B}	15.7 ± 1.2 ^{b,B,C}
	15	9.0 ± 1.6 ^{b,B}	16.2 ± 1.5 ^{a,C}	15.8 ± 3.8 ^{a,B}	13.0 ± 0.6 ^{a,C}

* Expressed in nmol Leu-p-NA/min·g cheese ± standard deviation; # expressed in nmol Lys-p-NA/min·g cheese ± standard deviation; P1 = 600 MPa, 6 min; P2 = 450 MPa, 6 min and P3 = 450 MPa, 9 min. Different non-capital letters (a, b, c, d) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C, D) in the same column indicate statistically significant differences among the same conditions ($p < 0.05$).

3.3. Effect of HPP on Textural Properties

Cheese texture is an important quality parameter that derives from the extensive chemical and biochemical changes that occur during ripening. Proteolysis is among the biochemical changes that may contribute to such textural properties. An evaluation of texture in Serra da Estrela cheese manufactured in different dairies was reported in [31].

The textural changes during the refrigerated storage of the control and HPP-treated *Serra da Estrela* cheeses are shown in Table 2. Significant texture changes ($p < 0.05$) were observed throughout refrigerated storage for all cheeses under analysis. However, HPP treatment did not significantly affect the hardness, consistency, adhesiveness, cohesiveness and gumminess of the cheeses at 0 months in comparison to the Ch_C cheeses ($p > 0.05$). Similarly, Garde et al. (2007) [12] and Delgado et al. (2012) [25] noticed that HPP (400 MPa/10 min) applied to La Serena cheese at 50 days of ripening and HPP (400 or 600 MPa/7 min) applied to Ibores cheese (raw goat's milk) at 30 days of ripening had no significant effect on texture. However, a significant effect was observed after storage (10 days) of HPP-treated and non-treated ripened (50 days) cheeses [22]. Camembert cheese (pasteurized cow milk) HPP-treated (550 MPa/10 min) at 45 days of ripening revealed no significant effect on firmness [32]. Hardness (instrumental/sensorial) values express the maximum force needed to attain a given deformation or the force required to compress a cheese with the molars to the point of penetration, respectively [33]. As shown in Table 2, the P1 cheeses became harder and more consistent during the storage period ($p < 0.001$), except for the samples measured after 15 months of storage. Such an increase may be related, on the one hand, with the progressively lower moisture contents reported up to 6 months of refrigerated storage. On the other hand, this variation in the textural properties of the P1 cheeses also revealed a positive correlation with the proteolysis levels previously discussed and with the sensorial analysis described below; as previously discussed, a lower ripening extension index (less primary proteolysis) is related to a firmer cheese. Delgado et al. (2013) [22] reported an increase in hardness for ripened Ibores cheeses treated with HPP (600 MPa/7 min) and after storage for 90 days. The P2 cheeses maintained similar values of hardness and consistency throughout storage ($p > 0.05$), with values closer to those of the Ch_C cheeses at 0 months (upon 45 days of ripening), in line with what was observed and discussed for the proteolytic indexes. In general, the cohesiveness increased during the first 90 days of storage for all the cheeses but then showed no significant differences ($p > 0.05$), except for the Ch_C cheeses at 15 months storage, which revealed significantly lower values ($p < 0.001$) than the HPP-treated cheeses. Cohesiveness (instrumental/sensorial) expresses the energy needed to be applied during mastication to break down the product until it is ready to be swallowed, representing the strength of internal bonds, or it measures the cohesion of the cheese mass throughout mastication, respectively [33]. Adhesiveness (instrumental/sensorial) is the work needed to overcome attractive forces between cheese and the textural plate, or it is evaluated by the degree to which the cheese adheres to tongue and mucosal tissues during the mastication progress, respectively [33]. The adhesiveness decreased in all the cheeses during storage, particularly for the HPP-treated P1 cheeses. A similar effect was verified by Delgado et al. (2015) [9] after 120 days storage of Casar cheeses, which had been previously ripened for 60 days and HPP-treated (600 MPa/5 and 20 min). HPP at 600 MPa (6 min) caused the most pronounced textural changes, increasing the gumminess when compared to the Ch_C cheeses. During the whole storage period, the P3 cheeses showed a similar gumminess to the Ch_C cheeses ($p > 0.05$), with an exception at 15 months ($p < 0.001$). Similar results were obtained for Ibores cheese treated by HPP (400 MPa/7 min at 60 days), with no significant effects on gumminess after 30 days of storage [22]. Gumminess (instrumental/sensorial) is a measure of the work needed to masticate the cheese to disintegrate it to a state ready for swallowing, or it expresses the number of chews that are required before the cheese is ready for swallowing, respectively [33].

Overall, and according to the literature, HPP had no immediate effect on the cheese texture parameters. However, during the storage time, some textural parameters tended to show some changes between the Ch_C and HPP-treated cheeses. Overall, lower differences in textural parameters were found for shorter storage periods and for the cheeses treated with HPP at a lower pressure intensity (P2).

Table 2. Textural properties of non-processed (Ch_C) and HPP-treated Serra da Estrela cheeses (P1, P2, P3).

Property	Storage Time (Months)	Ch_C	P1 600 MPa/6 min	P2 450 MPa/6 min	P3 450 MPa/9 min
Hardness (N)	0	0.16 ± 0.03 a,B,C	0.12 ± 0.03 a,C	0.12 ± 0.05 a,A	0.14 ± 0.04 a,C
	1.5	0.19 ± 0.04 a,b,B	0.22 ± 0.09 a,A,B	0.14 ± 0.07 b,A	0.20 ± 0.04 a,b,B
	3	0.12 ± 0.01 b,C,D	0.20 ± 0.02 a,B	0.10 ± 0.04 b,A	0.12 ± 0.04 a,C
	6	0.25 ± 0.08 a,A	0.28 ± 0.06 a,A	0.14 ± 0.02 b,A	0.26 ± 0.03 a,A
	15	0.11 ± 0.01 c,D	0.22 ± 0.04 a,A,B	0.12 ± 0.01 c,A	0.18 ± 0.03 b,B
Consistency (N/s)	0	0.96 ± 0.25 a,B,C	0.92 ± 0.33 a,C	1.1 ± 0.57 a,A	1.2 ± 0.47 a,C,D
	1.5	1.4 ± 0.37 b,c,B	1.8 ± 0.88 b,A,B	1.0 ± 0.65 c,A	2.7 ± 0.64 a,B
	3	0.72 ± 0.26 b,C,D	1.5 ± 0.19 a,B	0.68 ± 0.31 b,A	0.89 ± 0.34 b,D
	6	1.9 ± 0.76 a,A	2.1 ± 0.54 a,A	1.1 ± 0.14 b,A	1.9 ± 0.32 a,A
	15	0.67 ± 0.10 c,D	1.6 ± 0.28 a,A,B	0.78 ± 0.17 c,A	1.3 ± 0.25 b,B,C

Table 2. *Cont.*

Property	Storage Time (Months)	Ch_C	P1 600 MPa/6 min	P2 450 MPa/6 min	P3 450 MPa/9 min
Adhesiveness (N/s)	0	0.16 ± 0.08 ^{a,D}	0.16 ± 0.09 ^{a,D}	0.25 ± 0.18 ^{a,B,C}	0.15 ± 0.05 ^{a,C}
	1.5	0.36 ± 0.13 ^{a,b,B}	0.53 ± 0.27 ^{b,B,C}	0.26 ± 0.17 ^{a,B,C}	0.38 ± 0.12 ^{a,b,C}
	3	0.18 ± 0.05 ^{a,C,D}	0.48 ± 0.09 ^{c,C}	0.18 ± 0.10 ^{a,C}	0.30 ± 0.05 ^{b,C}
	6	0.82 ± 0.26 ^{b,A}	0.97 ± 0.26 ^{a,A}	0.47 ± 0.09 ^{b,A}	0.89 ± 0.19 ^{b,A}
	15	0.33 ± 0.06 ^{a,B,C}	0.72 ± 0.16 ^{b,B}	0.39 ± 0.06 ^{a,A,B}	0.68 ± 0.16 ^{b,B}
Cohesiveness (dimensionless)	0	0.56 ± 0.12 ^{a,B}	0.47 ± 0.06 ^{a,B}	0.56 ± 0.11 ^{a,B}	0.54 ± 0.19 ^{a,B}
	1.5	0.72 ± 0.14 ^{a,A}	0.73 ± 0.15 ^{a,A}	0.61 ± 0.24 ^{a,B}	0.61 ± 0.11 ^{a,B}
	3	0.68 ± 0.11 ^{a,A,B}	0.71 ± 0.12 ^{a,A}	0.73 ± 0.08 ^{a,A,B}	0.57 ± 0.08 ^{a,B}
	6	0.76 ± 0.09 ^{a,b,A,B}	0.73 ± 0.06 ^{b,A}	0.73 ± 0.09 ^{b,A,B}	0.82 ± 0.08 ^{a,A}
	15	0.012 ± 0.003 ^{b,C}	0.78 ± 0.07 ^{a,A}	0.87 ± 0.14 ^{a,A}	0.80 ± 0.09 ^{a,A}
Gumminess (N)	0	0.09 ± 0.03 ^{a,B,C}	0.06 ± 0.02 ^{a,C}	0.07 ± 0.04 ^{a,A}	0.07 ± 0.04 ^{a,C}
	1.5	0.14 ± 0.04 ^{a,A,B}	0.17 ± 0.09 ^{b,A,B}	0.10 ± 0.07 ^{b,A}	0.11 ± 0.04 ^{a,b,B}
	3	0.08 ± 0.01 ^{b,C}	0.14 ± 0.03 ^{a,B}	0.07 ± 0.04 ^{b,A}	0.07 ± 0.03 ^{b,C}
	6	0.16 ± 0.09 ^{a,A}	0.20 ± 0.04 ^{a,A}	0.10 ± 0.01 ^{b,A}	0.22 ± 0.03 ^{a,A}
	15	0.001 ± 0.000 ^{d,D}	0.17 ± 0.03 ^{a,A,B}	0.10 ± 0.03 ^{c,A}	0.14 ± 0.03 ^{b,B}

Different non-capital letters (a, b, c, d) in the same row indicate statistically significant differences between the same storage time, while different capital letters (A, B, C, D) in the same column indicate statistically significant differences among the same conditions ($p < 0.05$).

3.4. Effect of HPP on Sensorial Attributes

The results of the sensory evaluation of the *Serra da Estrela* cheeses are presented in Table 3. Some significant differences ($p < 0.05$) between the Ch_C and HPP-treated cheeses were found in terms of the appearance, odor, texture and taste attributes. Instrumental measurement of color showed that the color of the paste of the Ch_C cheeses became darker than the paste of the HPP-treated cheeses (less luminosity and less yellowness) [24] during storage; this was particularly observed for the sensory evaluation of color at 15 months of storage, for which significant differences ($p < 0.05$) were found between all the HPP-treated and Ch_C cheeses. Delgado et al. (2013) [22] also found that HPP-treated Ibores cheeses (raw goat's milk at 50 days of ripening at 400 and 600 MPa/7 min) were yellower than control cheeses. At 15 months of storage, the lower consistency (in paste appearance and texture) attributed to Ch_C relativity to the HPP-treated P1 cheeses was in agreement with the textural measurement, with the results showing that the HPP-treated P1 cheeses became harder and more consistent. The lactic, acid, animal and short-chain fatty acid odors did not significantly change for the P1 cheeses during the whole storage period ($p > 0.05$). Also, no significant differences in odor intensity between HPP-treated (400 or 600 MPa/5 min) and control raw ewe's [34,35] or cow's cheeses [36] during 10, 60 and 240 days of storage were reported. Significant odor differences between the Ch_C and HPP-treated cheeses were found for the P2 (acid and short-chain fatty acids) and P3 (acid) cheeses at 90 days storage; these results may derive from some particular heterogeneity of the samples or microbial endogenous enzyme activity.

No significant differences between the Ch_C and P3 cheeses ($p > 0.05$) were found in terms of texture attributes, showing that consistency and friability were not affected by HPP up to 15 months. Similarly, a HPP treatment (400 MPa/10 min at 50 days of ripening) on La Serena cheeses also revealed no significant effect on texture preference [12]. On the other hand, the P1 cheeses were perceived to be harder than the Ch_C cheeses at 15 months of storage ($p < 0.05$). Conversely, a hardness and friability decrease was observed for HPP-treated Ibores cheeses (600 MPa/7 min) in comparison to control cheeses [22]. Instrumental textural analysis revealed minor differences in the hardness and consistency of the P2 cheeses; however, these minor differences were perceived by the panel at 15 months storage ($p < 0.05$), where they considered the P2 cheeses softer compared to the Ch_C cheeses.

Table 3. Sensory ratings of attribute difference from control test: paired comparisons between blind control cheese (Ch_C) and HPP-treated *Serra da Estrela* cheeses (P1, P2, P3) at 0, 1.5, 6 and 15 months.

	Months	P1 vs. Ch_C	P2 vs. Ch_C	P3 vs. Ch_C
Appearance				
Colour	0	−0.38 ± 1.19	−0.88 ± 1.36	−0.25 ± 0.89
	1.5	−1.22 ± 2.28	−0.56 ± 1.74	−1.11 ± 2.15
	6	−0.90 ± 1.29	−0.60 ± 1.58	−1.00 ± 2.00
	15	−1.00 ± 0.82 *	−1.00 ± 1.05 *	−1.00 ± 0.94 *
Consistency	0	−1.43 ± 2.70	−1.29 ± 3.50	−0.43 ± 2.23
	1.5	0.89 ± 2.21	1.00 ± 2.18	0.67 ± 2.06
	6	1.50 ± 2.72	0.20 ± 2.74	−0.20 ± 1.87
	15	0.30 ± 1.06	−1.40 ± 1.27 *	−0.70 ± 1.06
Odour				
Lactic	0	−0.57 ± 1.13	0.00 ± 1.29	−0.29 ± 1.98
	1.5	−0.10 ± 1.52	−0.10 ± 2.42	0.50 ± 2.22
	6	−0.90 ± 2.89	−0.40 ± 1.35	−0.50 ± 1.43
	15	−0.30 ± 1.25	−0.20 ± 2.15	−0.60 ± 1.84
Acid	0	−0.75 ± 1.91	−0.13 ± 1.96	0.13 ± 1.73
	1.5	−0.30 ± 1.16	−2.10 ± 1.52 *	−1.80 ± 1.93 *
	6	0.50 ± 2.46	−0.20 ± 1.55	0.50 ± 1.43
	15	0.00 ± 1.00	1.00 ± 2.18	0.33 ± 1.23
Animal	0	0.43 ± 1.72	−0.71 ± 0.95	−0.29 ± 1.11
	1.5	−0.20 ± 1.62	−1.10 ± 2.08	−0.70 ± 2.00
	6	−0.60 ± 1.78	−0.10 ± 1.73	−0.20 ± 1.48
	15	−0.44 ± 0.73	−0.78 ± 1.09	−0.44 ± 1.01
SCFA	0	0.13 ± 1.73	0.13 ± 2.53	−0.38 ± 1.77
	1.5	−0.80 ± 1.40	−1.90 ± 1.52 *	−0.80 ± 1.87
	6	−0.78 ± 2.17	−0.44 ± 0.88	−0.22 ± 1.72
	15	0.60 ± 2.12	0.30 ± 3.65	−0.10 ± 2.23
Texture				
Consistency	0	−0.71 ± 1.25	−0.86 ± 0.90	0.00 ± 1.29
	1.5	0.11 ± 1.36	0.33 ± 1.80	0.67 ± 1.23
	6	1.30 ± 1.57	−1.10 ± 1.52	−0.40 ± 1.51
	15	1.00 ± 0.94 *	−2.00 ± 0.67 *	−0.20 ± 0.79
Friability	0	0.38 ± 1.06	0.13 ± 0.84	0.50 ± 1.07
	1.5	0.40 ± 1.17	0.40 ± 0.97	0.30 ± 0.68
	6	0.50 ± 0.85	−0.30 ± 0.68	−0.30 ± 0.68
	15	0.50 ± 1.08	−1.00 ± 1.70	−0.40 ± 0.70
Taste				
Salty	0	−0.71 ± 1.80	−1.00 ± 2.24	−1.14 ± 1.77
	1.5	0.00 ± 1.58	−0.22 ± 1.72	−0.89 ± 1.69
	6	−1.00 ± 1.66	−0.11 ± 0.93	−0.56 ± 1.74
	15	−1.89 ± 2.03 *	−0.56 ± 1.94	−0.11 ± 2.09
Acid	0	2.33 ± 0.56	1.19 ± 0.18	1.25 ± 0.79
	1.5	1.16 ± 0.09	0.92 ± 0.51	1.25 ± 0.47
	6	1.67 ± 0.45	1.87 ± 0.61	1.05 ± 0.76
	15	1.64 ± 0.58 *	1.99 ± 0.76 *	1.16 ± 1.00
Bitter	0	0.50 ± 1.60	−0.63 ± 1.41	−0.13 ± 1.36
	1.5	0.70 ± 0.71	0.20 ± 1.55	0.30 ± 1.77
	6	−0.44 ± 2.50	−0.33 ± 1.39	−0.11 ± 1.00
	15	−0.30 ± 2.44	0.20 ± 2.00	0.00 ± 1.49
After-taste	0	−0.50 ± 1.60	−0.38 ± 1.41	1.13 ± 1.36
	1.5	0.25 ± 0.71	0.13 ± 1.55	0.63 ± 1.77
	6	−0.67 ± 2.50	−0.22 ± 1.39	0.00 ± 1.00
	15	1.75 ± 2.44	0.00 ± 2.00	0.75 ± 1.49

Data expressed as mean (n = 10); * means significant difference ($p < 0.05$).

Relative to the flavor attributes, in general, the HPP-treated cheeses revealed a similar salty and acid taste ($p > 0.05$) than the Ch_C cheeses, being only significantly less intense for the P1 and P2 cheeses at 15 months ($p < 0.05$) in terms of acid taste and for P1 in terms of salty taste. Also, no significant differences in sour (sweet) and salty taste were denoted after 10 days of HPP (400 MPa/10 min at 50 days of ripening) of La Serena cheeses [12]. In our study, the bitter taste was not affected by HPP. However, according to the lower TCA/TN index observed in the HPP-treated cheeses, a less bitter flavor was expected for these cheeses [5]. The aftertaste attribute evidenced no effect of HPP ($p > 0.05$).

In general, few sensorial attributes were affected by HPP, with the P3 treatment (450 MPa/9 min) revealing minor differences in comparison to the Ch_C cheeses.

The lactic, acid, animal and vomit-like odors did not significantly change for the P1 cheeses during the whole storage period ($p > 0.05$). Also, no significant differences in odor intensity between HPP (400 or 600 MPa/5 min) and control raw ewe's [34,35] or cow's cheeses [36] during 10, 60 and 240 days of storage were reported. The sensory texture of the P3 cheeses was not affected by HPP, maintaining their consistency and friability attributes for 500 days relative to the hidden Ch_C cheeses ($p > 0.05$). Similar HPP treatment (400 MPa/10 min at 50 days of ripening) of La Serena cheeses also revealed no significant effects on texture preference [12]. On the other hand, the P1 cheeses revealed a harder and more friable paste at the sensorial level than the Ch_C cheeses, with this effect even more pronounced after 225 days of storage ($p < 0.05$). Contrarily, a hardness and friability decrease was verified for HPP Ibores cheeses (600 MPa/7 min) in comparation to control cheeses [22]. Instrumental textural analysis revealed minor differences in the hardness and consistency of the P2 cheeses; however, these minor differences were significantly denoted by the panel at 500 days ($p < 0.05$), where they considered the P2 cheeses softer in a sensorial pair comparison relative to the hidden Ch_C cheeses. This different evaluation, instrumental versus sensorial, can probably be attributed to the sampling analysis. Cheese sensorial analyses were performed using cheeses slices, as cheese is consumed, which allowed for an evaluation of all the cheese zones (near to the rind and the cheese interior). On the other hand, the texture analysis evaluated cylindric samples (crossed rind to rind), with each sample representing different cheese zones, and this led to a great variation in the cheese texture parameters.

Relative to the flavor attributes, in general, the HPP-treated cheeses revealed similar salty and acid tastes ($p > 0.05$), being only significantly less intense in the P1 and P2 cheeses at 500 days ($p < 0.05$). Also, no significant differences in sour, sweet and salty tastes were denoted after 10 days of HPP (400 MPa/10 min at 50 days of ripening) of La Serena cheeses [12]. In our study, the bitter taste was not affected by HPP. However, according to the lower TCA/TN index in the HPP-treated cheeses, a less bitter flavor was expected for these cheeses [5]. The aftertaste attribute changed during storage, but there was no evidence that HPP had an effect ($p > 0.05$).

In summary, few attributes were affected by HPP, with the P3 treatment (450 MPa/9 min) revealing minor differences in comparison to the Ch_C cheeses.

4. Conclusions

High-pressure processing of 45-day-old ripened Serra da Estrela cheese at 450–600 MPa for 6–9 min affected the evolution of cheese proteolytic indexes throughout a 15-month storage period; the cheeses treated at 600 MPa registered lower ripening extension and depth indexes than the cheeses treated at 450 MPa (6 or 9 min). Aminopeptidase activities were not closely related to the cheese proteolysis trends; an inverse relationship was observed between the proteolytic indexes and aminopeptidase activity during storage for all cheeses, except for those treated at 600 MPa, during the first 6 months of storage. High-pressure treatment affected textural changes throughout storage; higher proteolysis indexes (associated with a more intense primary and secondary proteolysis) were associated with softer textures (HPP at 450 MPa for 6 or 9 min), whereas lower proteolysis indexes (HPP at 600 MPa for 6 min) were associated with a harder texture by 15 months of storage, yet they were closer to that of the conventional *Serra da Estrela* cheese upon 45 days of ripening (optimum conditions). High-pressure processing affected the color of the cheese paste, which was lighter than that of the control cheeses. However, very few sensory characteristics were affected by HPP, which were mainly related to consistency and hardness, whereas flavor and odor qualities were not greatly affected; the treatment at 450 MPa for 9 min revealed the least differences in comparison to the Ch_C cheeses.

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Abbreviations

The following abbreviations are used in this manuscript:

HPP	High-pressure processing
PDO	Protected denomination of origin
WSN	Water-soluble nitrogen
TCA	Trichloroacetic-acid-soluble nitrogen
PTA	Phosphotungstic-acid-soluble nitrogen
TN	Total nitrogen content
WSN/TN	Water-soluble nitrogen to total nitrogen
TCA/TN	Ripening depth index
PTA/TN	Free amino acid index

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