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High Pressure Processing of Raw Ewe's Cheese Promotes Microbiological Safety and Quality During Prolonged Storage

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Abstract

Serra da Estrela cheese (a raw ewe's milk) ripened for 45 days was treated at 600 MPa for 6 min (P1) and at 450 MPa for 6 (P2) and 9 min (P3) and kept in refrigerated storage for 15 months. *Lactobacillus* and *Lactococcus* viable cell numbers were reduced in 3.2–3.6 and 2.7–3.6 log cycle units, respectively. Lower reductions were verified for total aerobic mesophilic and *Enterococcus* viable cell numbers in cheeses treated at 450 MPa (2.4–2.5 and 1.2 log reductions, respectively). In HPP cheeses, yeasts and moulds were below the enumeration limit up to 6 months of storage, but at 15 months 3.6–4.2 log cfu/g were quantified in all cheeses, while *Enterobacteriaceae* were inactivated to below the quantification limit. The increment of pressure treatment caused a greater impact on the microbiota's viability than the increase in time under pressure. During storage, minor total colour differences were determined for HPP P3 cheese surface relative to control cheeses, Ch_C, at 45 days of ripening. HPP can thus be a good process to apply after cheese manufacture, since it offers a good potential to render raw-milk cheese microbiologically safer, with minimal changes in quality.

Keywords: *Serra da Estrela* cheese; microbial evolution; moisture; protein; fat

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1. Introduction

The origin of *Serra da Estrela* cheese, a traditional Portuguese raw ewe's milk cheese with Protected Denomination of Origin (PDO) certification, has been reported back to as early as the Roman occupation of the Iberian Peninsula [1,2]. Even though its productivity has witnessed significant increases over the centuries, the millennial manufacturing process has changed very little, consecrating its genuine authenticity and high quality. In fact, manufactured from raw ewe's milk, salt and a crude extract of vegetable rennet (dried thistle flowers of *Cynara cardunculus* L.), *Serra da Estrela* cheese may be considered, from a technological point of view, the "parent" of other important national PDO ewe's milk cheeses such as the *Azeitão* or *Serpa* cheeses [1].

The uniqueness of raw milk cheeses relies on the necessary dynamic interaction between diverse native microbiota that together drive the necessary biochemical reactions

during the ripening period toward the development of optimum and unique aroma, flavor and texture profiles [3]. With a minimum ripening period of 30 days, *Serra da Estrela* cheese is generally consumed as a semi-soft cheese [4], where its appreciated cheese texture is influenced by the cardoon flowers used [5,6]. Lactic acid bacteria (LAB), in particular *Lactobacillus* spp. and *Lactococcus* spp., have been reported as the predominant bacterial groups in *Serra da Estrela* cheese [1,3,7–10], but other groups of microorganisms, such as *Enterobacteriaceae*, *Staphylococcus* spp. and yeasts have also been found [7,9–11]. Furthermore, when hygiene practices are insufficient, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Hafnia alvei*, *Staphylococcus xylosus*, *Staphylococcus epidermidis* and *Enterococcus faecium* may also be found in this cheese type [7,12].

Given its uniqueness, *Serra da Estrela* cheese is a symbol of national gastronomic heritage that has crossed borders and is sought by consumers worldwide; this status demands that its shelf-life be as prolonged as possible, on the one hand, and, on the other, that its singular and unique sensory quality is not altered during transport and storage. The demand for such microbiologically safe, wholesome, tasty and minimally processed foods has led to the search for new production strategies, including novel food processing technologies. High-pressure processing (HPP) has been highlighted as a non-thermal technology for food processing that can produce microbiologically safe food products, with minimal changes to their characteristics, demonstrating clear advantages over thermal processing [13]. In cheese production, HPP may be applied directly to milk for subsequent cheese production [14,15], after pressing of the curd and/or during cheese ripening These applications have different objectives, including the improvement of cheese preservation or the deceleration/acceleration of the ripening process [13,16,17]. For example, HPP has been applied to Torta del Casar raw ewe's milk cheese either as a procedure to prevent over-ripening [18], to minimize biogenic amine build-up [19] or off-odours [20], to improve the microbiological quality and increase cheese safety [21] or to extend the commercialization period [22]. In the case of other raw ewe's milk (La Serena, Castellano type, *Serra da Estrela* cheeses), raw goat's (Ibores cheese) or cow's milk cheeses, HPP has mainly been used to improve the cheese's microbiological quality and increase cheese safety and shelf-life [21–28]. In all these research studies, after HPP treatment the cheeses were ripened and/or stored for periods between 14 and 240 days. Taking advantage of the selective inactivation effect of HPP on microorganisms, its application to cheese, with minimal effects on quality and without changing the traditional manufacture procedure, and so with potential to keep the PDO status, as is the case with *Serra da Estrela* cheese, is of great interest. In a previous study, where HPP was applied to small, 15 g portions of *Serra da Estrela* cheese, results revealed that LAB were the microorganisms least affected by HPP (0.86 log cycle reductions), while *Enterobacteriaceae*, *L. innocua* (inoculated at 8.56 log cfu/g) and yeasts and moulds were reduced to below the limit of quantification during 100 days of refrigerated storage [24].

Based on the above rationale, HPP can potentially be used as a non-thermal pasteurisation process for raw milk cheeses to assure microbial safety and increase shelf-life, enabling ready-to-go-to-market potential. The main objective of this work was to study the effect of HPP on 45-day ripened PDO Serra da Estrela cheese (optimally organoleptic, already meeting PDO sensory standards and thus ready for commercial release), immediately after HPP and during post-processing refrigerated storage (similar to retail-like refrigeration for 15 months).

In the present work we applied three high-pressure processing (HPP) conditions—600 MPa/6 min (P1), 450 MPa/6 min (P2) and 450 MPa/9 min (P3)—chosen to probe the influence of pressure intensity and holding time, the two parameters known to modulate

HPP efficacy [29]. Microbiological and physicochemical changes were monitored immediately after pressurization and during 15 months of refrigerated storage. These measurements are part of a large, integrated data-set that includes (i) the lipidomic study (tri-, di- and monoglycerides, esterified and non-esterified fatty-acid profiles, and conjugated linoleic acid) and the derived nutritional indices (atherogenicity, thrombogenicity) [30]; (ii) the proteolysis/texture/sensory study (lower ripening-depth indexes and with few sensory attributes revealing significant differences between control and HPP cheeses) described previously [31]; and (iii) the microbiological safety and quality data presented here.

To our knowledge, this is the first longitudinal investigation in which raw ewe's milk cheeses are evaluated exhaustively and in parallel for safety, lipid stability, proteolytic evolution, texture and sensory performance over such an extended, 15-month cold-storage period. By capturing all major quality attributes in a single experimental design, the study provides an unprecedented evidence base for the economic valorization and internationalization of PDO *Serra da Estrela* cheese processed by HPP.

2. Materials and Methods

2.1. Cheese Manufacture

One hundred and fifty litres of *Bordaleira* ewe's milk were collected in the morning at two farms in the PDO region, Portugal. Milk was transported under refrigeration (30 min) and kept under refrigeration in a reservoir with constant mixing until cheese manufacture. Two batches of *Serra da Estrela* PDO cheese were manufactured; due to the limited production capacity, one was manufactured in the morning (Batch A) and the other after lunch (Batch B) (Figure 1). Briefly, raw ewe's milk, which was simply filtered through a clean, fine white cloth to remove impurities like hair and dust, was coagulated via an aqueous extract of thistle flower (cardoon, 0.3 g/L), without the addition of any commercial starter culture. After coagulation, the curd was cut and worked in moulds [2]. Fifty-six cheeses (of about 0.5 kg each) were manufactured and ripened (first 15 days at 9 ± 2 °C and 90% RH and then at 11 ± 2 °C and 75% RH) at the dairy over 45 days according to the traditional procedures [1] in order to reach the optimum organoleptic quality. During the ripening period, the cheeses were washed weekly and turned regularly. Upon ripening, the cheeses were placed into polyamide–polyethylene (PA-PE) bags (Plásticos Macar–Indústria de Plásticos Lda, Santo Tirso, Portugal) and vacuum sealed (vacuum packaging machine, HenkoVac E-193, Aveiro, Portugal). The sealed cheeses were then transported, under refrigeration, to the laboratory at University of Aveiro for HPP (transport took about 1 h and 30 min).

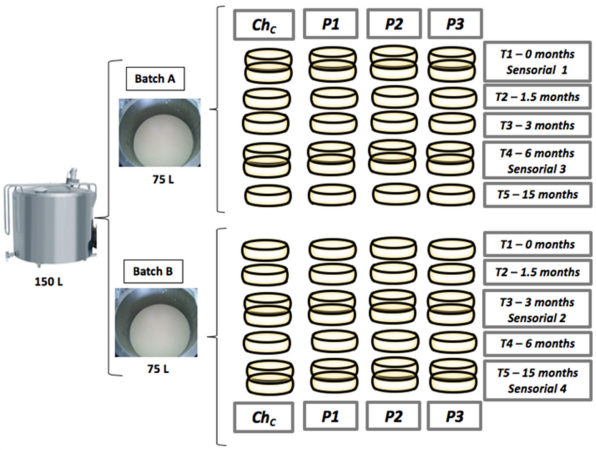

Figure 1. Schematic representation of cheese production and sampling (600 MPa/6 min (P1), 450 MPa/6 min (P2) and 450 MPa/9 min (P3) and Ch$_c$ refers to control cheeses).

2.2. High-Pressure Processing

Treatments were performed in a 55 L capacity, industrial-scale, high-pressure device (model 55, Hyperbaric, Burgos, Spain). Batches of 7 *Serra da Estrela* cheeses were subject to one of three HPP treatments (two batches per treatment (A and B)): 600 MPa/6 min (P1), 450 MPa/6 min (P2) and 450 MPa/9 min (P3) (Figure 1). The goal was to assess the effect of moderate (450 MPa) and high pressure (600 MPa), along with the influence of treatment duration. Average times to reach 450 and 600 MPa were 1.42 and 2.30 min, and depressurisation times 3 and 5 s, respectively. The initial temperature of the water used as transmitting fluid was 8 °C and remained under 24 °C throughout processing. The two manufactured batches (A and B) were processed into different high-pressure processing batches and used as sampling replicas. Upon HPP, control and HPP-treated cheeses (56 in total) underwent vacuum packaging and were placed in refrigerated storage (4 °C) in a storage chamber with minimal variations in humidity and light.

2.3. Sampling

In order to characterize the milk used for cheese manufacture, two milk samples (one from the morning batch and another from the afternoon batch) were collected from the refrigerated reservoir. After coagulation, cutting and pressing of the curd, fresh cheese samples were collected (1.5 h after milk coagulation initiated), one per cheese batch. In the case of the ripened cheeses, 4 cheeses were taken from each batch (one per treatment and one control) at each sampling time and were analysed; a total of 8 cheeses were analysed, plus 4 cheeses for sensorial analysis, per sampling point, at 0, 1.5, 3, 6 and 15 months of storage as shown in Figure 1. Thus, 0 month therefore represents the day of HPP (i.e., 45 days after manufacture). Subsequent sampling points (1.5, 3, 6 and 15 months) refer to time elapsed in refrigerated storage after HPP. Aliquots of each cheese (≈35 g per sample) were stored at −80 °C until physicochemical analyses were carried out. Non-processed cheeses were used as controls (Ch_C).

2.4. Microbiological Analyses

Cheeses were cut in half, and a thin slice was cut through the innermost, the intermediate and the outermost layers of the cheese; the rind was removed, and a combination of all three was mixed to obtain a single 10 g cheese sample. This sample was aseptically handled and homogenized for 4 min using 90 mL of 2% (w/v) aqueous sodium citrate solution as extraction buffer in a Stomacher Lab-Blender 400 (Seward, Milano, Italy). Aliquots of 1.5 mL were then taken and decimally diluted in 13.5 mL of sterile 0.1% (w/v) aqueous peptone and then plated in triplicate on several culture media. The following microbial groups were enumerated, using the pour-plate method: *Enterobacteriaceae* on violet–red bile dextrose agar (VRBDA from Merck, Darmstadt, Germany); coliforms and *E. coli* on chromocult coliform agar (CCA from Merck) both incubated at 37 °C for 1 d. The spread-plate technique was used for enumeration of *Enterococcus* spp. on kanamycin aesculin azide agar base (KAAA from Oxoid, UK) and incubated at 37 °C for 1 d; *Lactobacillus* spp. on Man, Rogosa and Sharpe (MRS from Merck) and incubated at 30 °C for 3 d; *Lactococcus* spp. on M17 (Liofilchem, Roseto degli Abruzzi, Italy) and incubated at 30 °C for 3 d; and *Bacillus* spp. on HiChrome (from Fluka, Mumbai, India) and incubated at 30 °C for 2 d. The Miles and Misra technique [32] was used for enumeration of total aerobic mesophilic microorganisms on plate count agar (PCA from Merck) and incubated at 30 °C for 3 d; total anaerobic microorganisms on PCA and incubated at 37 °C for 2 d in anaerobic jars (Merck) with Merck Anaerocult A (Merck); total psychotrophic microorganisms on PCA and incubated at 20 °C for 5 d; yeasts and moulds on rose-bengal chloramphenicol agar (RBCA from Merck) and incubated at 25 °C for 5 d; *Staphylococcus* spp. on Baird-Parker

agar (BPA from Merck) with egg yolk tellurite emulsion (Liofilchem) and incubated at 37 °C for 2 d; *Listeria* spp. on PALCAM agar selective agar base (Liofilchem), with selective supplement for PALCAM (Liofilchem) and incubated at 37 °C for 2 d; and *Pseudomonas* spp. on pseudomonas agar base (PAB from Liofilchem) with glycerol and pseudomonas CFC supplement (CFC from Liofilchem) and incubated at 30 °C for 2 d. Petri dishes containing 30–300 and 10–100 colony forming units (cfu) were selected for counting for spread plate and pour plate, and Miles and Misra, respectively.

The results were converted into logarithmic decimals of the number of cfu per g of cheese sample, and values were considered below the limit of quantification of 2.0 log cfu/g for the spread plate and pour plate techniques and 3.0 log cfu/g for Miles and Misra technique. Less than 1 log cfu/mL was considered for milk samples due to direct liquid sample plating.

2.5. Physicochemical Analyses

The pH was measured at room temperature in random points of cheeses using a properly calibrated pH/temperature penetration pH meter (Testo 205, Testo, Inc., West Chester, PA, USA). The titratable acidity was determined according to AOAC Official Method 920.124 [33], by titration to a pH value of 8.9 using an automatic titrator with pH meter (Crison–Titromatic 1S with pH electrode 50 14, Barcelona, Spain). Measurements of water activity were performed using a Novasina LabSwift water activity (a_w) analyser (Lachen, Switzerland), by direct reading at room temperature after proper stabilization. Moisture content was determined by drying 2 g of cheese to a constant weight (ca. 24 h) at 105 °C using laboratory oven drying equipment (Venticell, MMM Medcenter Einrichtungen GmbH, Munich, Germany). All these physicochemical analyses were performed in triplicate per cheese sample. Fat content was determined, in duplicate, by the method of Gerber following the Portuguese Standard Protocol NP-2105 [34] and ISO 3433 [35] using a butyrometer calibrated in a range from 0 to 40% fat. The total nitrogen (TN) content was determined by the micro-Kjeldahl procedure using a Kjeltec system 1002 Distilling unit (Tecator, Sweden) and the crude protein content determined by multiplying the total nitrogen content by 6.38 [36].

2.6. Colour

Colour parameters were measured using a Minolta Konica CM 2300d (Konica MinoltaCM 2300d, Osaka, Japan) at room temperature. The colour parameters were recorded in CIE Lab system and directly computed through the original SpectraMagic NX software (Konica Minolta, Osaka, Japan), according to the International Commission on Illumination regulations. Cheeses were kept for 1 h at room temperature before measurements. The colour parameters L^*, a^* and b^* were measured in each cheese surface/rind and core/interior. The total colour difference (ΔE^*) was calculated using Equation (1):

$$\Delta E^* = \left[(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2 \right]^{1/2} \quad (1)$$

where ΔE^* is the total colour difference between a sample and the control (initial values at 45 days of ripening); b^* and L_0^* are the lightness of the sample and respective control; a^* and a_0^* are the redness of the sample and control, respectively; and b^* and b_0^* are the yellowness of the sample and control, respectively. In addition, chroma (Equation (2)) and hue angle (Equation (3)) were also recorded:

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

$$^{\circ}h = arctg\left(\frac{b^*}{a^*}\right) \quad (3)$$

Measurements were performed selecting six random spots, and read in triplicate per cheese section (surface and core).

2.7. Statistical Analyses

Analysis of variance (ANOVA) was performed to establish the effect of different processing conditions (three HPP treatments and control—Ch_C), the effect of storage and the combined effect of processing conditions and storage. The significant difference Bonferroni test was applied to compare the mean values of parameters, with the significance assigned at $p \leq 0.05$. Mean values and standard error of mean are reported. SPSS software version 24.0 was used for the statistical analysis.

3. Results and Discussion

3.1. Changes in Serra da Estrela Cheese Microbial Composition Induced by HPP

Across all microbial groups analysed, a consistent pattern was observed: control cheeses (ChC) showed the highest viable cell numbers throughout storage, while HPP treatments significantly reduced microbial loads, with the strongest effect seen at 600 MPa (P1) (Figure 2 and Table 1). In general, there were no significant differences between P2 and P3, indicating that extending treatment time from 6 to 9 min at 450 MPa did not enhance microbial inactivation ($p > 0.05$).

Table 1. Log reductions of HPP cheeses relative to control Serra da Estrela cheese at 0 months of refrigerated storage.

	P1 600 MPa/6′	**P2 450 MPa/6′**	**P3 450 MPa/9′**
Lactobacillus spp.	3.55	3.20	3.47
Lactococcus spp.	3.60	2.71	3.41
Enterococcus spp.	4.93	1.21	1.24
Total aerobic microorganisms	5.32	2.40	2.48
Total anaerobic microorganisms	4.34	1.44	1.51
Total psychotropic microorganisms	4.35	1.11	1.11
Enterobacteriaceae	>5.9	>5.9	>5.9
Coliforms	3.05	0.36	0.28
Escherichia coli	>1.1	>1.1	>1.1
Staphylococcus spp.	6.39	4.37	4.55
Pseudomonas spp.	4.74	4.74	4.74
Bacillus spp.	4.68	2.13	2.32
Yeasts and moulds	>1.1	>1.1	>1.1

3.1.1. Lactic Acid Bacteria, Enterococcus, Total Aerobic Mesophilic, Anaerobic and Psychotropic Bacteria

Lactic acid bacteria (LAB), *Enterococcus* and total microbiota viabilities were significantly affected by HPP, the storage time and by the combination of both ($p \leq 0.05$).

Figure 2 shows the effect of HPP on the *Serra da Estrela* cheese microbiota, immediately following HPP and throughout the 15-month storage period.

For *Lactobacillus* and *Lactococcus* (Figure 2a and b, respectively), high counts were observed at 45 days of ripening (≈9.5–9.8 log cfu/g), in line with values reported in Serra da Estrela cheese [9,10,24]. Despite these high viable cell numbers, immediately following HPP the *Lactobacillus* counts decreased by 3.2–3.6 log cycles, while *Lactococcus* spp. were slightly more baroresistant, particularly at 450 MPa. In a previous *Serra da Estrela* cheese study (using small cheese portions), a lower *Lactobacillus* reduction (0.82 log cycle) for cheese HPP at 600 MPa for 3 min was reported [24]. We can highlight the main difference between the previous study and this study in the cheese presentation: portions (first study) vs. whole cheese (present study). In the previous first study only four cheeses were used, whose rind was removed, paste homogenized and which was divided into several portions (15 g) and vacuum-packed. In contrast, the present study involves whole cheeses (500 g with rind) at each sampling time, which is a real case-study much closer to the typical conservation process. Casar cheeses (also produced from raw ewe's milk) with 5 weeks of ripening HPP-treated at 400 and 600 MPa/5 min showed 1.64 and 6.51 log cycle reductions in *Lactobacillus* counts, immediately after pressurization [19]. Based on these trends, the results obtained in this study at 600 MPa corroborate those reported in the literature, whereas at 450 MPa a higher impact was observed in this study; in general, log cycle reductions were 2-fold higher. During storage, these LAB declined by about 1 log cycle in Ch_C and up to 2 log cycles in P1 cheeses.

Enterococcus, an important flavour-associated group, remained stable in Ch_C (≈7 log cfu/g) but showed a >4.9 log reduction with P1 treatment, while P2 and P3 caused milder reductions (~1.2 log) (Figure 2c and Table 1). Similar results were reported in Casar ewe's cheese (with 5 weeks of ripening) treated at 400 and 600 MPa/5 min (≈0.6 and ≈4.6 log cycle units) [19].

In general, the viable cell numbers of total aerobic mesophilic, anaerobic and psychotropic microorganisms in Ch_C cheeses remained stable over storage without significant differences ($p > 0.05$), as can be observed in Figure 2d–f. Similar total aerobic counts and behaviour (8–10 log) have already been reported at 35 days of ripening [11] and for 100 days of storage [24]. Total aerobic, anaerobic and psychrotrophic microorganisms showed modest reductions at 450 MPa (1.1–2.4 log), without significant differences between P2 and P3 cheeses ($p > 0.05$), and strong reductions at 600 MPa (4.3–5.3 log) ($p < 0.001$). A previous study on pieces of *Serra da Estrela* cheese revealed that HPP caused lower reductions (0.47–1.20 log cycles) independently of the pressure treatment applied [24]. Likewise, 1.29 and 1.44 log cycle reductions were reported for the total microbiota after similar HPP treatments in Casar cheese [21] and in La Serena cheeses [26]. On the other hand, another study performed on Casar cheese reported 1.33 to 4.43 log cycle reductions in total viable cell numbers after HPP at 400 and 600 MPa/5 min at 35 days of ripening [19], which are aligned with those observed in the present work. Furthermore, Delgado et al. (2012) [28] studied the effect of HPP (500 MPa/7 min) on Ibores cheeses (raw goat's milk), and also verified a significant decrease in viable cell numbers of psychrotrophic microorganisms and at a similar order of magnitude (1.1 log cycle reductions) at 50 days of ripening.

3.1.2. Contaminant Microbial Groups

The evolution of the studied microbial contaminants of Serra da Estrela cheese is depicted in Figure 3. At 45 d of ripening, control *Serra da Estrela* cheeses (Ch_C) revealed viable cell numbers between 6–9 log cfu/g for all contaminant microbial groups tested except for *E. coli*. Over storage time, viable cell numbers remained stable for coliforms and *Bacillus* ($p > 0.05$) but decreased to levels $\leq$ 4 log cfu/g for *Enterobacteriaceae*, *Pseudomonas* spp. and *Staphylococcus* spp. ($p < 0.001$). The loss in viability over time may possibly be due

to competition between existing species [9]. The literature reports a large decrease in viable cell numbers of *Enterobacteriaceae* in this cheese over ripening [3,24].

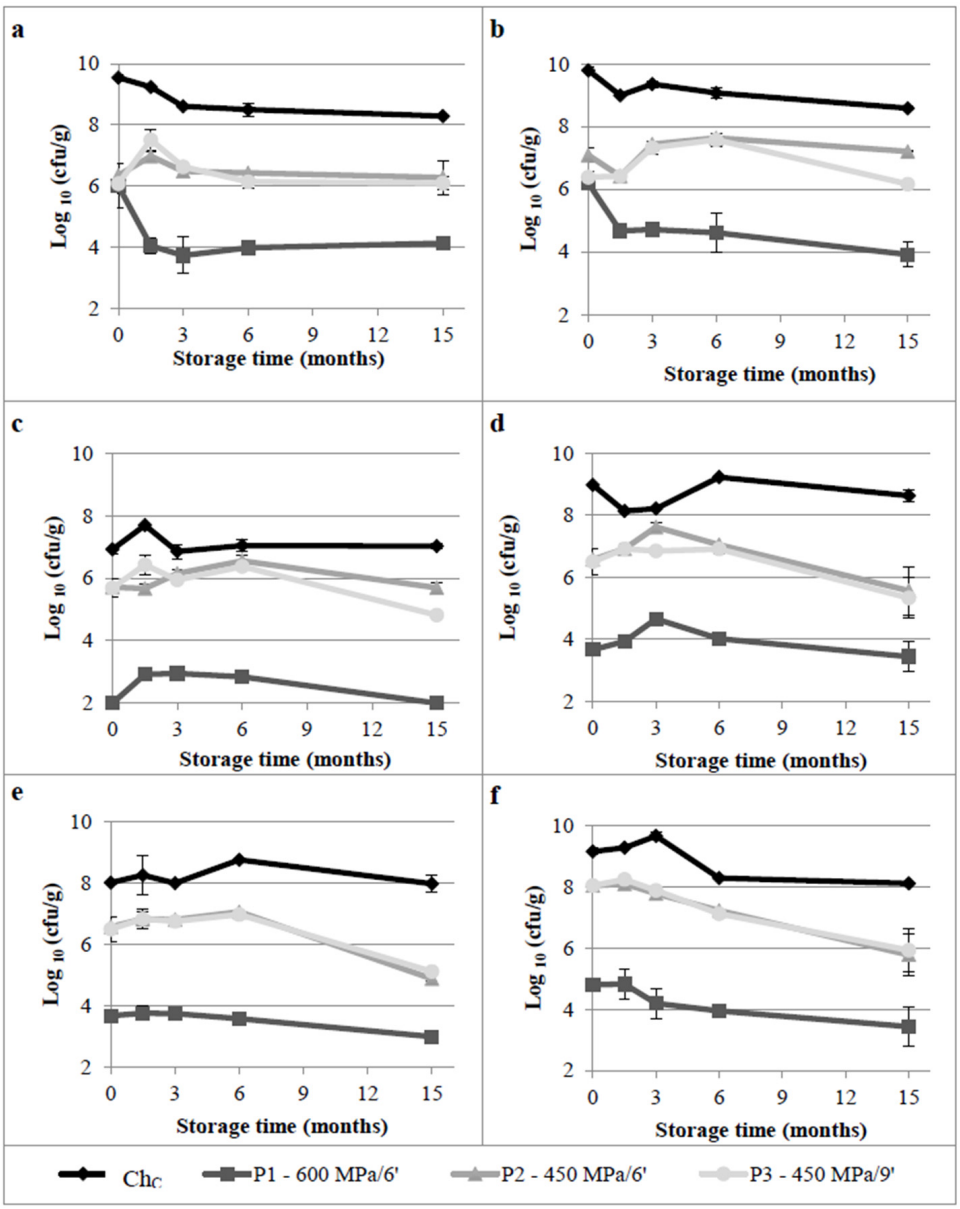

Figure 2. Viable cell counts of (**a**) *Lactobacillus* spp., (**b**) *Lactococcus* spp., (**c**) *Enterococcus* spp., (**d**) total aerobic, (**e**) anaerobic and (**f**) psychrotrophic microorganisms in control (Ch_C) and HPP (P1, P2 and P3) Serra da Estrela cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage (4 °C). Empty symbols represent microbial loads below the quantification limit.

Independently of the microbial group considered, and the associated microbial load present at 45 days, HPP treatment was able to effectively reduce viability either to levels below enumeration limit in the case of *Enterobacteriaceae*, *E. coli* and *Pseudomonas* spp. across all three treatments and remained stable throughout the storage period, or to 3–5 log cycles lower in the case of coliforms or *Bacillus* sp. in P1 treated cheese and *Staphylococcus* spp. in all P1, P2 and P3 cheeses.

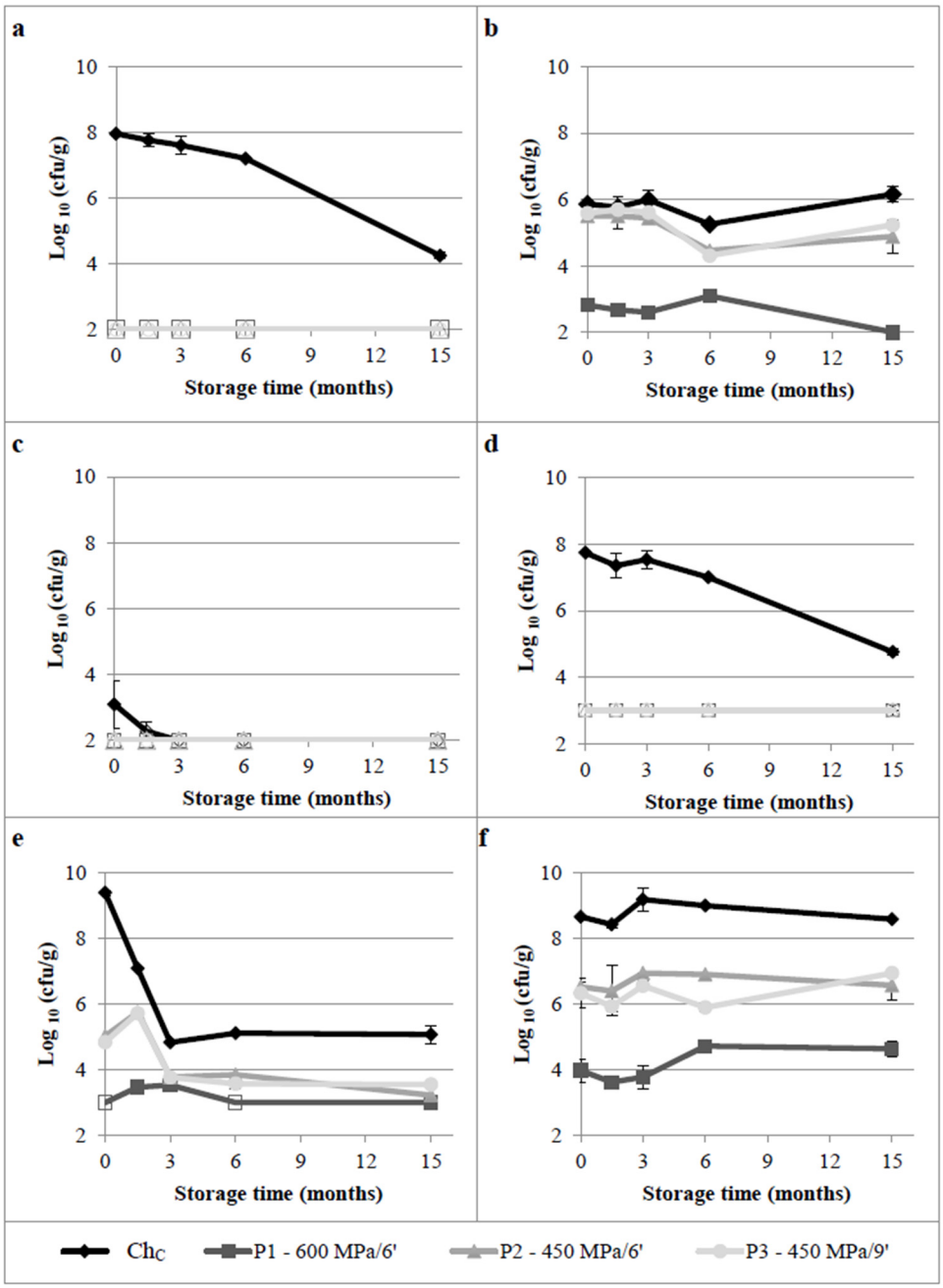

Figure 3. Viable cell counts of (**a**) Enterobacteriaceae, (**b**) total coliforms, (**c**) *E. coli*, (**d**) *Pseudomonas* spp., (**e**) *Staphylococcus* spp. and (**f**) *Bacillus* spp. in control (Ch_C) and HPP (P1, P2 and P3) *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage. Empty symbols represent microbial loads below the quantification limit.

HPP treatment at 450 MPa, independently of application time (P2 and P3), promoted a milder effect than treatment at 600 MPa ($p < 0.01$), in the case of coliforms, *Staphylococcus* and *Bacillus*. The significant decrease in viable cell numbers ($p < 0.01$) achieved by HPP at 450 MPa (about 0.3, 4.4 and 2.2 log cycles, respectively) was incremented further at 600 MPa (3, 6.4 and 4.7 log cycles, respectively).

This overall behaviour is not always corroborated by similar studies with raw ewe's milk cheeses reported in the literature. For example, minor reductions in *Enterobacteriaceae* (2.29 log cycles) or in *Pseudomonas* spp. (2.5 log cycles) were achieved in Torta del Casar cheese after HPP at 600 MPa/5 min at 60 days of ripening [21]. In La Serena cheeses, while minor reductions in *Enterobacteriaceae* (1.98 log cycles) were also reported for cheeses after

HPP at 400 MPa/10 min at 50 days of ripening, high log cycle reductions (>5 log cycles) were reported for coliforms [26]. Calzada et al. (2023) [19] reported similar high log cycle reductions in total coliforms (>3.5 log to counts below the limit) in La Serena cheese after HPP at 400 and 600 MPa/5 min at 35 days of ripening.

An important observation is related to viable cell numbers of *Staphylococcus*. The European Commission [37] has established *Staphylococcus* as a microbiological criterion (process-hygiene criteria) for coagulase-positive *Staphylococcus* to be analysed for cheeses made from raw milk. In Ch_C cheeses, general *Staphylococcus* viable cell numbers decreased 4 log cycles to 4.83 ± 0.00 log cfu/g from 45 days of ripening—0 months to 3 months of storage, remaining constant thereafter ($p > 0.05$). The presence of *Staphylococcus* at 45 days of ripening in Ch_C cheeses was above the established limit of 10^5 cfu/g [37]; previous studies have also reported levels above the required threshold at a similar ripening stage (6.60 log cfu/g at 42 days of ripening) of *Serra da Estrela* cheese [38].

Although a high total count of *Staphylococcus* was observed after 45 days of ripening, the absence of specific enumeration of coagulase-positive *Staphylococcus* limits the direct assessment of compliance with regulatory standards; however, the literature indicates that the predominant Staphylococcus species found in curd are *Staph. xylosus*, *Staph. aureus* and *Staph. epidermidis*, and at lower proportions, *Staph. simulans* and *Staph. hominis* [7], with the predominant species in dairy products being typically coagulase-negative, thus suggesting a lower potential risk. Furthermore, more recently, Rocha et al. (2023) [39] reported that assessment of hygienic and food safety microbiological indicators confirmed that Serra da Estrela cheeses are a safe food product. Despite the high viable cell numbers of general *Staphylococcus* at 45 days of ripening, HPP treatment enabled a significant decrease ($p < 0.001$) of between 4 and 6 log cycles in cheeses treated at 450 MPa (P2, P3) or at 600 MPa (P1) (to below the enumeration limit). Cheeses treated at 600 MPa showed values below the enumeration limit or near the quantification limit (3 log cfu/g) during the 15 months of storage. However, based on the European Commission, (2005) [37], only the cheese subjected to HPP could be consumed at 45 days of ripening; from 3 months of storage onwards, all cheeses could be consumed, including Ch_C cheeses. Similar results were reported by [19,23], where, following HPP at 600 MPa/5 min (at 35 days of ripening and during storage for 240 days), *Staphylococcus* viable cell numbers were reduced to below the enumeration level. *Listeria* spp. were verified below the enumeration limit in all cheeses during all storage periods.

3.1.3. Yeasts and Moulds

Yeasts and moulds were present at around 4 log cfu/g throughout the 15 months of storage (Figure A1) in Ch_C cheeses ($p > 0.05$). Yeast loads reported in the literature for *Serra da Estrela* cheese are variable, ranging in average between 3 and 6 log cfu/g, depending greatly on ripening and storage temperature and relatively humidity [7,9,11,24]. HPP caused a reduction to below the quantification limit (<3 log cfu/g equivalent to >0.8 log reduction) in all treated cheeses and throughout the 6 months of storage. An identical effect was verified in a previous *Serra da Estrela* cheese study (using small cheese portions), although greater reductions were observed (≥3.6 log cycles, initial value of 5.58 log cfu/g) [24]. Rodríguez-Pinilla et al. (2015) [21] reported 2.05 log cycle reductions of moulds in Casar cheese after HPP at 600 MPa/5 min at 60 days of ripening [21]. At 15 months of storage, all cheeses revealed similar viable cell numbers of yeasts and moulds (3.6–4.2 log cfu/g), without significant differences in comparison to Ch_C cheeses ($p > 0.05$).

3.1.4. Overview of the Effect of HPP on Microbiota and Cheese Quality

As previously mentioned, the different microbial groups tested felt the impact of HPP differently, having expressed different reduction profiles at different pressure intensity, as observed in Table 1. The inactivation mechanisms induced by HPP have been correlated to cell damage in membranes, which are thought to be a primary target for HPP, in addition to enzyme denaturation and changes in cell morphology [40]. HPP above 300 MPa induces irreversible denaturation of enzymes and proteins, alterations that have been shown to lead to ribosome dissociation, which may lead to limited cell viability [41]. In general, the changes in the different microbial groups induced by HPP are in agreement with those reported in the literature. The prokaryotic microorganisms (bacteria) showed a higher resistance towards pressure than eukaryote microorganisms (yeasts and moulds) [42]. In general, Gram-positive bacteria (*Lactococcus*, *Lactobacillus*, *Bacillus*, *Staphylococcus*) were more resistant, reflecting lower cycle reductions, than Gram-negative bacteria (*E. coli* and *Pseudomonas*) [43], which were reduced to below the quantification limit. This difference in resistance can be correlated with the thicker peptidoglycan layer in Gram-positive microorganisms, which has generally been shown to be more pressure-resistant [40,43,44], but it also suggested that Gram-negative cell membrane complexity causes higher susceptibility to HPP [45]. Among LAB bacteria, morphological differences seem to influence resistance to HPP. In general, coccoid-shaped LAB, such as *Lactococcus* species, reveal greater resistance to HPP compared to rod-shaped LAB like *Lactobacillus* [46]; post-HPP treatment, coccoid Lactococcus generally showed higher survival rates than rod-shaped Lactobacillus This difference is likely due to the lower surface area-to-volume ratio of coccoid cells, which reduces membrane exposure to pressure-induced damage. Rod-shaped bacteria, by contrast, are more susceptible to structural damage due to their elongated form, which is exposed to greater mechanical stress at the poles and along their length. In addition to the direct microbial reductions induced by HPP, the selective inactivation of spoilage-related Gram-negative bacteria and *Staphylococcus* spp. appears to have reshaped the post-pressurization microbiota in a way that is neutral, or even favourable, to flavour development. The persistently high counts of LAB and *Enterococcus* spp. provide the enzymatic framework for controlled proteolysis and lipolysis, while the lowered levels of competing Gram-negative groups limit the risk of off-flavours. This interpretation agrees with the descriptive sensory data obtained by trained panellists: lactic, acidic, animal and short-chain-fatty-acid odours did not differ significantly ($p > 0.05$) between P1 cheeses and non-processed controls throughout 15 months of storage [31].

These microbial shifts can also be influenced by the biochemical and physical properties of the cheese [31]. Under P1 treatment (600 MPa/6 min), the reduced microbial activity was associated with marked deceleration of proteolysis, evidenced by the maintenance of the WSN/TN index within 27–30% throughout storage—closely mirroring the value of the control cheese at 45 days of ripening pre-HPP, a stage typically regarded as the sensory optimum. In contrast, under moderate HPP conditions (P2: 450 MPa/6 min; P3: 450 MPa/9 min), *Enterococcus* spp. remained viable and may have continued to contribute with its enzymatic activity to the cheese's properties. As a result, P2 cheeses retained textural firmness and consistency values comparable to the control, while P3 cheeses displayed sensory attributes virtually indistinguishable from untreated samples at the end of storage [31].

Lipidomic analyses further demonstrated that HPP had no significant impact on the cheese's lipid profile, regardless of pressure condition. Levels of triglycerides, esterified and free fatty acids, and conjugated linoleic acid (CLA) remained stable across treatments, as did the nutritional indices of atherogenicity (IA $\approx$ 2.3) and thrombogenicity (IT $\approx$ 2.6), highlighting the preservation of both compositional integrity and nutritional value [30].

Taken together, these findings indicate that HPP selectively modulates the cheese microbiota in a way that influences ripening dynamics while maintaining sensory and nutritional quality. By eliminating undesirable Gram-negative and hygiene-indicator bacteria and preserving key functional bacterial groups such as LAB and *Enterococcus*, HPP influences proteolysis, acidification and textural development. The most intense treatment (P1) effectively "locks" the cheese at its ideal ripening stage, extending shelf-life without compromising safety or sensory quality, while the moderate treatments (P2, P3) offer a more gradual evolution, preserving the traditional sensory profile with enhanced microbiological safety. This microbiota-driven modulation of cheese quality parameters confirms the functional importance of microbial viability and balance in the long-term stability of traditional raw milk cheeses.

These results underscore the fact that the post-HPP microbiota profile not only determines microbial safety but also plays a central role in shaping the biochemical progression, textural integrity and sensory preservation of *Serra da Estrela* cheese over long-term storage.

3.2. Changes in Physicochemical Characteristics Induced by HPP

3.2.1. Moisture, Fat and Protein Contents

As expected, the moisture content significantly decreased during 45 days of ripening from 59.7 ± 2.3 in curd to 47.8 ± 1.1 for Ch_C cheeses ($p < 0.001$). The decrease in moisture content was caused by natural and progressive water evaporation due to the relative humidity in the ripening chambers [47]. Values inside the range 40.1–48.4% were reported by Correia et al. (2026) [48] and Macedo et al. (2004) [38] at 42 days of ripening. At 45 days (0 months of storage), no significant differences were found between the moisture content of Ch_C and all HPP-treated cheeses ($p > 0.05$). No significant differences were found between Ch_C and P3 cheeses throughout 15 months of storage ($p > 0.05$). The results obtained in the present work are in agreement with those reported in the literature, showing that the HPP caused no changes in the moisture content [13]. For example, in Casar cheeses, Delgado et al. (2015) [22] showed no significant differences in moisture content between control and HPP cheeses, despite the natural decrease during storage.

As far as the fat content is concerned, HPP had no significant ($p > 0.05$) effect on fat content values as listed in Table 2; values between 25 and 27% were registered. Similar values have been reported for this type of cheese [49–51]. Delgado et al. (2012) [28] also found no significant differences in the fat content of control and HPP Ibores cheeses.

Table 2. Moisture, fat, protein content, pH values, titratable acidity and water activity measured in control (Ch_C) and HPP (P1, P2, P3) *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage.

	Ch_C	**P1—600 MPa/6′**	**P2—450 MPa/6′**	**P3—450 MPa/9′**
Water Content	% (*w*/*w*) STD	% (*w*/*w*) STD	% (*w*/*w*) STD	% (*w*/*w*) STD
0	47.8 ± 1.10 ^{b,A}	49.4 ± 0.20 ^{a,A}	47.8 ± 0.89 ^{b,A}	48.0 ± 0.82 ^{b,A}
1.5	46.0 ± 0.31 ^{a,B}	45.8 ± 0.58 ^{a,B}	45.8 ± 1.02 ^{a,B}	45.4 ± 1.17 ^{a,B}
3	44.5 ± 0.86 ^{a,C}	44.1 ± 0.41 ^{a,D}	44.8 ± 0.18 ^{a,B,C}	44.4 ± 0.26 ^{a,B,C}
6	43.6 ± 1.18 ^{b,C}	44.5 ± 0.49 ^{a,b,C,D}	45.1 ± 0.37 ^{a,B,C}	43.9 ± 1.00 ^{a,b,C}
15	44.5 ± 0.21 ^{a,b,C}	44.9 ± 0.52 ^{a,C}	44.4 ± 0.56 ^{a,b,C}	43.9 ± 0.71 ^{b,C}
Fat Content	% (*w*/*w*) STD	% (*w*/*w*) STD	% (*w*/*w*) STD	% (*w*/*w*) STD
0	26.1 ± 1.25 ^{a,B}	24.9 ± 1.97 ^{a,B}	25.4 ± 1.31 ^{a,B}	26.5 ± 1.47 ^{a,A}
3	27.4 ± 0.25 ^{a,A,B}	28.0 ± 0.71 ^{a,A}	27.9 ± 0.25 ^{a,A}	28.4 ± 1.11 ^{a,A}
6	28.3 ± 1.19 ^{a,A}	28.9 ± 1.60 ^{a,A}	26.5 ± 0.41 ^{a,A,B}	27.1 ± 1.49 ^{a,A}
15	28.1 ± 0.48 ^{a,A}	27.6 ± 0.48 ^{a,A,B}	27.9 ± 1.77 ^{a,A}	27.9 ± 0.75 ^{a,A}

Table 2. *Cont.*

	Ch_C	P1—600 MPa/6′	P2—450 MPa/6′	P3—450 MPa/9′
Protein Content	% (*w*/*w*) STD	% (*w*/*w*) STD	% (*w*/*w*) STD	% (*w*/*w*) STD
0	22.0 ± 0.25 ^{b,B}	22.6 ± 0.15 ^{a,b,B}	23.5 ± 0.29 ^{a,A}	20.4 ± 0.32 ^{c,C}
3	23.3 ± 0.19 ^{a,A}	22.4 ± 0.44 ^{b,c,B}	23.1 ± 0.19 ^{a,b,A}	21.8 ± 0.56 ^{c,B}
6	23.7 ± 0.40 ^{a,A}	23.1 ± 0.48 ^{a,A,B}	22.8 ± 0.41 ^{a,A}	23.5 ± 0.55 ^{a,A}
15	22.5 ± 0.29 ^{b,B}	23.7 ± 0.33 ^{a,A}	23.5 ± 0.34 ^{a,A}	23.8 ± 0.60 ^{a,A}
pH values	pH STD	pH STD	pH STD	pH STD
0	5.36 ± 0.17 ^{A,a}	5.27 ± 0.17 ^{B,a}	5.24 ± 0.01 ^{B,a}	5.23 ± 0.02 ^{B,a}
1.5	5.39 ± 0.07 ^{A,a}	5.34 ± 0.01 ^{A,a}	5.33 ± 0.02 ^{A,a}	5.36 ± 0.06 ^{A,a}
3	5.25 ± 0.04 ^{A,a}	5.22 ± 0.01 ^{C,a,b}	5.19 ± 0.02 ^{C,b}	5.21 ± 0.01 ^{B,C,b}
6	5.29 ± 0.02 ^{A,a}	5.17 ± 0.01 ^{D,b}	5.17 ± 0.01 ^{C,b}	5.15 ± 0.03 ^{C,b}
15	5.26 ± 0.02 ^{A,a}	5.22 ± 0.01 ^{C,b}	5.17 ± 0.01 ^{C,b}	5.18 ± 0.05 ^{B,C,b}
Titratable acidity	$g_{lactic\ acid}$/100 gSTD	$g_{lactic\ acid}$/100 g STD	$g_{lactic\ acid}$/100 g STD	$g_{lactic\ acid}$/100 g STD
0	0.69 ± 0.01 ^{E,a}	0.83 ± 0.09 ^{D,a}	0.81 ± 0.18 ^{D,a}	0.80 ± 0.09 ^{E,a}
1.5	1.09 ± 0.06 ^{D,a}	1.00 ± 0.10 ^{C,a}	1.03 ± 0.03 ^{C,a}	1.02 ± 0.09 ^{D,a}
3	1.27 ± 0.07 ^{C,a}	1.17 ± 0.05 ^{B,C,b}	1.18 ± 0.06 ^{C,a,b}	1.21 ± 0.05 ^{C,a,b}
6	1.50 ± 0.10 ^{B,a}	1.20 ± 0.09 ^{B,b}	1.63 ± 0.06 ^{B,a}	1.47 ± 0.17 ^{B,a}
15	1.94 ± 0.06 ^{A,a}	1.61 ± 0.16 ^{A,b}	1.84 ± 0.10 ^{A,a}	1.88 ± 0.09 ^{A,a}
Water activity	a_w STD	a_w STD	a_w STD	a_w STD
0	0.959 ± 0.002 ^{a,A}	0.959 ± 0.004 ^{a,A}	0.957 ± 0.001 ^{a,A}	0.956 ± 0.002 ^{a,A}
1.5	0.954 ± 0.005 ^{a,A,B}	0.953 ± 0.001 ^{a,B}	0.952 ± 0.002 ^{a,B}	0.951 ± 0.001 ^{a,B}
3	0.953 ± 0.001 ^{a,b,B}	0.954 ± 0.001 ^{a,A,B}	0.950 ± 0.003 ^{c,B}	0.951 ± 0.001 ^{b,c,B}
6	0.950 ± 0.002 ^{b,B}	0.954 ± 0.001 ^{a,A,B}	0.951 ± 0.000 ^{b,B}	0.950 ± 0.000 ^{b,B}
15	0.928 ± 0.004 ^{b,C}	0.935 ± 0.006 ^{a,C}	0.932 ± 0.003 ^{a,b,C}	0.933 ± 0.004 ^{a,b,C}

Different non-capital letters (a, b, c) in the same row indicate statistically significant differences between treatments at a given storage time, while different capital letters (A, B, C, D, E) in the same column indicate statistically significant differences across storage times within a single treatment ($p \leq 0.05$).

Ch_C cheeses revealed 22.0 ± 0.25% (*w*/*w*) of protein content at 45 days of ripening, which had significantly increased to 23.7 ± 0.40% ($p < 0.001$) up to 6 months, as shown in Table 2. A similar protein content (22.1% (*w*/*w*)) was determined by Macedo and Malcata (1997) [51] at 60 days of ripening, but lower values were recently reported by [48] (14.69–19.35% (*w*/*w*)). HPP affected protein content in different ways; at 45 days, treatment P1 revealed no significant difference ($p > 0.05$) whereas treatments P2 and P3 led to a higher or lower protein content ($p < 0.01$), respectively, in comparison to Ch_C cheeses. Similarly, in Ibores cheeses, Delgado et al. (2012) [28] quantified a high protein content in cheeses that had been HPP-treated at 400 MPa/7 min and significantly lower in cheeses treated with HPP at 600 MPa/7 min at 50 days of ripening. In the present study, at 15 months of storage, no significant differences were found between HPP cheeses ($p > 0.05$), yet protein contents were significantly higher compared to Ch_C cheeses ($p \leq 0.05$).

3.2.2. pH Values and Titratable Acidity

The milk's average pH values were 6.69 ± 0.07, which decreased to 6.53 ± 0.06 in curd/fresh cheese. At 45 days of ripening, 0 months of storage, cheese pH values had decreased to 5.36 ± 0.17, remaining fairly constant over 15 months of storage, particularly from the 1.5-month sampling point onwards ($p > 0.05$), as shown in Table 2. The pH variations observed are naturally related to the microbiota's metabolism, mainly due to the production and consumption of lactic acid during cheese ripening [7,11,52]. Similar pH values have been reported in the literature (4.82–5.66) [24,38,53,54]. HPP did not affect initial pH values; at 0 months, no significant differences between Ch_C and HPP-treated cheeses

were reported for pH and titratable acidity (TA) values ($p > 0.05$). The pH values of HPP cheese were maintained within limits reported for Ch_C cheeses (5.23 and 5.39) throughout storage, except at 6 months, where a slight decrease was observed in comparison to Ch_C cheeses ($p < 0.001$). Martínez-Rodríguez et al. (2012) [13] found that pressure treatments modify the pH to an extension degree that differs according to treatment conditions and cheese age, and that the pH differences between HPP and untreated samples fade out as the ripening process progresses. In the present study, although no significant differences were found in the first days of storage, significant differences were verified toward the end of the storage period (Table 2), but without biological relevance since they were less than 0.1 units of pH. Calzada et al. (2024) [18] reported a similar behaviour, with control cheeses revealing higher and significantly different pH values in relation to HPP cheese, only from month 4 onwards. Evolution of TA values was, in general, correlated with pH values; lower pH values corresponded to higher TA values. Over 15 months of storage, TA values increased steadily and significantly ($p < 0.001$) to values between 1.61–1.94 g lactic acid/100 g (fresh curd had 0.18 g lactic acid/100 g). Lower TA values were determined for P1 cheeses, with significant differences ($p \leq 0.05$) at 6 and 15 months relative to other cheeses. *Serra da Estrela* cheese acidification depends on endogenous microbial viable cell numbers [1]. Thus, this trend may be correlated with the number and type of microbiota present, which was higher and more diverse in Ch_C cheeses than in HPP cheese samples (P1 cheeses underwent the highest reduction in bacterial viable numbers), as well as with the possible reduced ability to produce acid compounds induced by HPP [13], resulting in very low TA values. A similar behaviour was reported in a previous study of small portion samples of *Serra da Estrela* cheese [24].

3.2.3. Water Activity

As expected, water activity (a_w) values depended on storage time ($p < 0.001$) in both Ch_C and HPP treated cheeses; a_w decreased in all cheeses from to 0.928 at 15 months for Ch_C cheeses, and from 0.956–0.959 to 0.932–0.935 at 15 months for HPP cheeses (Table 2). Ávila et al (2016) [23] reported a decrease in a_w for HPP and control raw ewe cheeses over 60 days of ripening. In the present study, for 3 months, no significant a_w differences were found between all cheeses, similarly to what had already been reported in the previous study of 100 days of storage [24]. During the whole storage period no significant a_w differences ($p > 0.05$) were observed between Ch_C and P3 cheeses.

3.2.4. Colour

Colour analysis is a relevant parameter in understanding the HPP and storage time impacts on the visual appearance of the cheese's surface, but also in the cheese's core (Figure 4). *Serra da Estrela* cheeses are recognized by the slightly yellowish colour, which can be instrumentally measured by the CIE b^* values. In this study, it was shown that HPP had an impact on both cheese surface and cheese core; HPP cheeses showed higher CIE b^* values, more yellowness, than the Ch_C cheeses throughout the storage period. The cheese surface CIE b^* values decreased slightly until the end of storage (Table A1). The b^* value variations measured on the surface of Ch_C cheeses (19.77 and 22.78) over 15 months were similar to those that Correia et al. (2026) [48] reported (19.55–22.17). A similar behaviour was reported by Delgado et al. (2013) [27] and Delgado et al. (2015) [22] for goat and ewe cheeses, respectively.

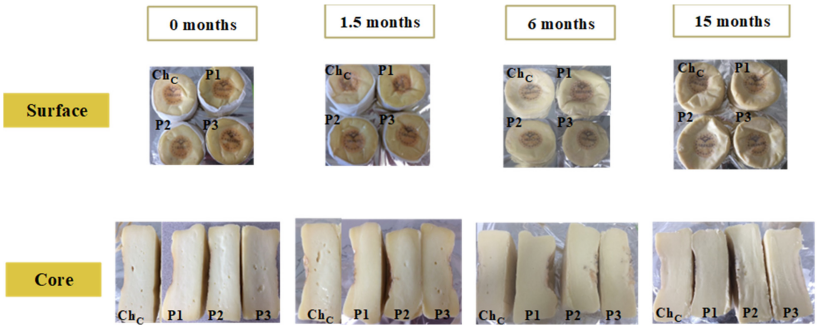

Figure 4. Visual appearance of control (Ch$_C$) and HPP (P1, P2 and P3) *Serra da Estrela* cheese at 0, 1.5, 6 and 15 months of refrigerated storage.

Total colour difference (ΔE^*) was calculated relative to Ch$_C$ cheeses at 45 days, with the results being expressed in Figure 5a for cheese surface and 5b for cheese core. For the cheese surface, a total difference of 8.03 to 13.68 for Ch$_C$ cheeses and 7.38 to 15.52 for HPP cheeses was verified. HPP P3 cheeses revealed lower total differences, but the HPP P1 cheeses showed higher values. Minor total differences were quantified in the cheeses' cores. In addition, determination of chroma (C^*) revealed that the HPP cheeses had higher values on associated surface and core, indicating that HPP led to a superior cheese colour intensity, which decreased during storage in cheese surface and increased in cheese core (Figure A2A,B), corroborating CIE b^* value trends. The hue degree (°h) values were higher in HPP cheeses (fluctuating between 90.78 and 94.24 for cheese surface and 94.24–97.36 for cheese core); values near to 90° correspond to a visual yellowish colour. Ch$_C$ cheeses showed similar values (ranging from 90.01 to 91.95 for cheese surface and 93.17–94.07 for core, (Figure A2C,D) during storage.

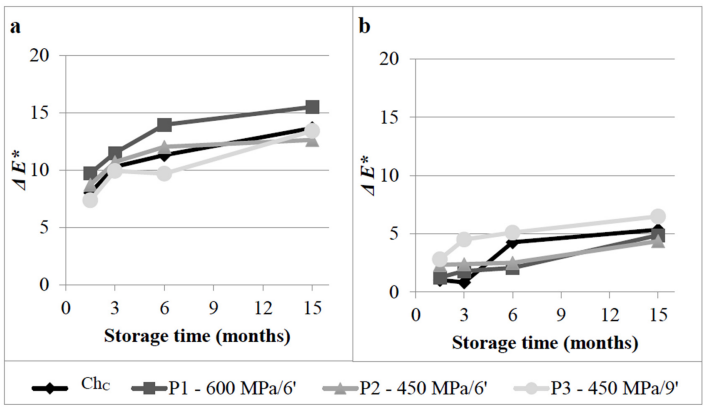

Figure 5. Total colour differences in (**a**) cheese surface and (**b**) cheese core measured comparatively to control (Ch$_C$) and HPP (P1, P2 and P3) *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage.

According to the literature, the colour variations induced by HPP can be correlated with changes in moisture content and/or with modifications in the protein matrix and/or due to proteolytic alterations [22].

4. Conclusions

The results obtained in this work demonstrate the promising effect of application of HPP in enhancing the microbial safety of *Serra da Estrela* cheese (a raw ewe's milk cheese) throughout extended storage (15 months shelf-life) without jeopardizing physicochemical quality. The nature of the HPP treatment applied was revealed to be significantly different: despite a few exceptions, in general, a longer period (6 min vs. 9 min at 450 MPa) was

not significant among all the parameters tested, whereas pressure intensity (450 MPa vs. 600 MPa) was. In terms of microbial viable cell numbers, higher log cycle reductions were achieved in P1 cheeses treated at 600 MPa/6 min. If a minimal impact on microbial population with important metabolic activity for *Serra da Estrela* cheese (*Lactobacillus*, *Lactococcus*, *Enterococcus*) is sought, while simultaneously inactivating pathogenic microorganisms, an intermediate pressure intensity could be the best treatment to be applied. Concerning cheese quality, interesting results were also obtained. HPP P2 cheeses showed similar moisture and fat content and higher protein content in comparison to Ch_C cheese at month 0. Instrumental colour analysis revealed higher *b** values (more yellowness) in HPP cheeses' surface and core than in control cheeses, even though all became whitened due to vacuum packaging. Overall, the HPP treatment of *Serra da Estrela* cheese proposed and studied herein proved appropriate to control the microbiological—including safety hazards—biochemical, textural and sensory changes that occur over extended storage. Enhancement of the fundamental knowledge regarding relationships between HPP and cheese technological and biochemical features was also accomplished, enabling a rational improvement in the quality and safety control of artisanal *Serra da Estrela* cheese.

In fact, if minimal impact on the microbial population with important metabolic activity for cheese ripening (*Lactobacillus*, *Lactococcus*, *Enterococcus*) is to be achieved, while simultaneously inactivating contaminant and/or pathogenic microorganisms, and maintaining the artisanal cheese's physicochemical and quality parameters, an intermediate pressure intensity could be the best treatment to be applied, i.e., 525 MPa/6 min.

However, it is important to note that current regulations under the PDO specification for *Serra da Estrela* cheese do not allow the use of HPP or other technological interventions. Nonetheless, the depth and consistency of the results obtained in this study provide a strong scientific basis to support future dialogue and potentially justify modifications to the specification. This integration could only be considered if it is unequivocally demonstrated that the distinctive characteristics of this traditional cheese remain unaltered.

To that end, a more robust and comprehensive validation effort—involving multiple certified dairies, producers, and the regulatory and certification bodies that safeguard the PDO identity of *Serra da Estrela* cheese—is essential to ensure that the distinctive sensory and physicochemical characteristics of the cheese are preserved following HPP treatment. Collaborative research like this would not only confirm the reproducibility of the results across different production contexts but also generate shared knowledge and evidence capable of informing the regulatory framework. Ultimately, this process would benefit all stakeholders in the sector by enhancing food safety assurance, supporting shelf-life extension, and potentially opening up new market opportunities for this emblematic artisanal product.

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Data Availability Statement: The data presented in this study are openly available in Institutional Repository of the Universidade Católica Portuguesa at https://repositorio.ucp.pt/entities/publication/3b5e48d8-f119-493c-84df-eefcd7c43a8d or http://hdl.handle.net/10400.14/32137, being one Chapter of the PhD Thesis of the first author, which is a standard procedure for PhD theses in Portugal. [Universidade Católica Portuguesa] [http://hdl.handle.net/10400.14/3213] [10400.14/32137].

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

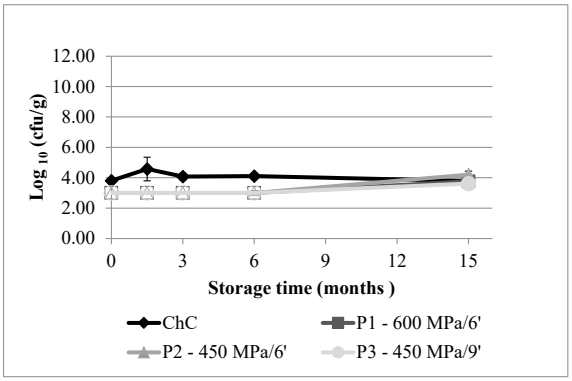

Figure A1. Yeasts and moulds viable cell numbers in Serra da Estrela cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage, of control cheeses (Ch_C) and HPP cheeses (P1, P2 and P3). Empty symbols represent microbial loads below the quantification limit (3 log cfu/g).

Table A1. Colour CIE parameters measured in cheese surface and cheese core of Serra da Estrela cheese at cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage, of different HPP treatments and non-processed cheeses (Ch_C).

			Ch_C	P1—600 MPa/6′	P2—450 MPa/6′	P3—450 MPa/9′
		Months	**STD**	**STD**	**STD**	**STD**
Cheese Surface Colour	*L**	0	68.44 ± 0.57	67.60 ± 2.62	69.11 ± 0.75	69.61 ± 1.44
		1.5	76.25 ± 1.11	77.00 ± 0.53	77.43 ± 1.18	76.60 ± 1.23
		3	78.57 ± 1.10	78.78 ± 1.02	79.43 ± 1.23	79.18 ± 1.25
		6	79.66 ± 1.10	81.34 ± 1.07	80.56 ± 0.52	78.87 ± 0.62
		15	81.78 ± 1.23	81.59 ± 1.01	81.03 ± 1.15	82.32 ± 0.98
	*a**	0	0.00 ± 0.20	−0.35 ± 0.48	−0.76 ± 0.21	−0.45 ± 0.53
		1.5	−0.71 ± 0.17	−1.75 ± 0.17	−0.84 ± 0.53	−1.46 ± 0.16
		3	−0.39 ± 0.36	−1.21 ± 0.77	−0.68 ± 0.42	−1.04 ± 0.41
		6	−0.37 ± 0.32	−1.04 ± 0.35	−0.32 ± 0.64	−0.61 ± 0.19
		15	−0.30 ± 0.26	−0.58 ± 0.50	−0.56 ± 0.40	−0.84 ± 0.22
	*b**	0	22.78 ± 0.20	25.74 ± 0.90	25.31 ± 1.10	25.27 ± 0.65
		1.5	21.01 ± 0.17	23.66 ± 0.63	22.68 ± 0.37	23.16 ± 0.62
		3	20.98 ± 0.36	23.19 ± 1.19	22.45 ± 1.20	22.66 ± 1.09
		6	21.38 ± 0.32	23.35 ± 1.20	21.60 ± 1.15	22.39 ± 1.72
		15	19.77 ± 0.26	19.04 ± 2.73	21.12 ± 2.07	20.97 ± 1.11

Table A1. *Cont.*

			Ch_C	P1—600 MPa/6′	P2—450 MPa/6′	P3—450 MPa/9′
Cheese Core Colour	*L**	0	85.62 ± 0.57	86.56 ± 1.40	86.83 ± 2.47	89.11 ± 1.70
		1.5	85.14 ± 1.11	85.55 ± 3.63	84.75 ± 1.53	86.71 ± 1.63
		3	85.76 ± 1.10	85.52 ± 1.01	85.10 ± 1.40	85.12 ± 2.60
		6	83.52 ± 1.10	85.04 ± 1.58	85.05 ± 1.27	84.62 ± 0.93
		15	83.25 ± 0.74	82.18 ± 0.91	83.03 ± 0.70	82.97 ± 0.85
	*a**	0	−1.19 ± 0.20	−2.72 ± 0.08	−2.65 ± 0.16	−2.66 ± 0.11
		1.5	−1.31 ± 0.17	−2.56 ± 0.16	−2.56 ± 0.06	−2.76 ± 0.14
		3	−1.32 ± 0.36	−2.66 ± 0.08	−2.59 ± 0.05	−2.59 ± 0.07
		6	−1.37 ± 0.32	−2.17 ± 0.21	−2.38 ± 0.16	−2.59 ± 0.02
		15	−1.24 ± 0.07	−1.73 ± 0.19	−2.14 ± 0.07	−2.03 ± 0.13
	*b**	0	17.49 ± 0.81	21.38 ± 0.46	20.94 ± 0.48	20.56 ± 0.41
		1.5	18.40 ± 1.86	22.12 ± 0.61	22.01 ± 0.65	22.04 ± 0.81
		3	18.30 ± 1.37	22.86 ± 0.44	22.60 ± 0.52	22.69 ± 0.84
		6	21.20 ± 1.10	22.69 ± 0.26	22.71 ± 0.60	23.01 ± 0.60
		15	22.29 ± 0.34	23.29 ± 0.35	23.03 ± 0.45	22.60 ± 0.28

CIE *L** significantly increased ($p < 0.05$) over 500 days of storage in cheese surface of all cheeses, from 67.60–69.61 to 81.03–82.32, as shown in Table A1. In the present study, Serra da Estrela cheese rinds were revealed to be more luminous than cheeses analysed by Correia et al. (2016) [48] (56.61 to 61.36). On the other hand, in the cheese core, the *L** values slightly decreased for all cheeses during 500 days of cheese storage, being more accentuated for HPP P3 (presented higher *L** value at 45 days of ripening). Thus, the storage led to a lightness increase for cheese surface and a decrease for cheese core, in each case without differences at 500 days between NonP and HPP at 450 MPa (P2 and P3). Results similar to the present work were reported by Delgado et al. (2015) [22] and [55] in the interior of ewe and cow raw cheese. CIE *a** was lower on cheese surface and core in HPP cheeses, with more redness than NonP cheeses over 500 days of storage time (Table A1). Delgado et al. (2015) [28] also reported lower *a** values in HPP (600 MPa/5min) Casar cheeses than controls.

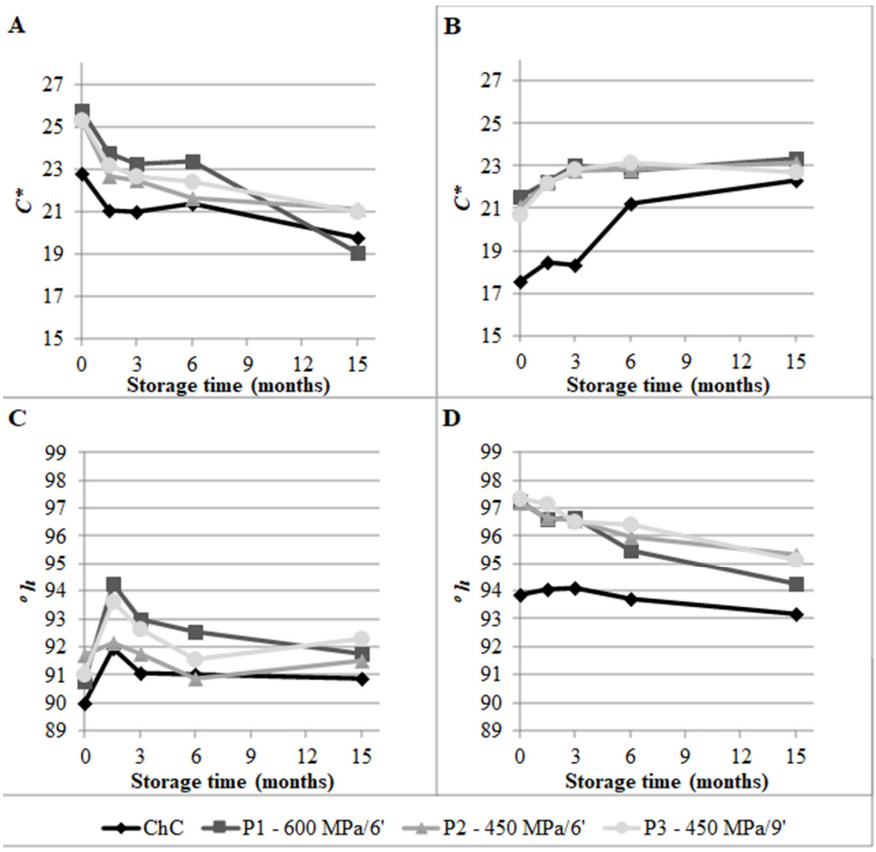

Figure A2. Chroma in (**A**) cheese surface and (**B**) cheese core; and hue degree in (**C**) cheese surface and (**D**) cheese core calculated comparatively to non-treated *Serra da Estrela* cheese at 0, 1.5, 3, 6 and 15 months of refrigerated storage of control cheeses (Ch_C) and HPP cheeses (P1, P2 and P3).

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