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RESEARCH ARTICLE

Characteristics and proteolysis of a Spanish blue cheese made with raw or pasteurised milk

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Valdeón cheese is a Spanish Protected Geographical Indication of blue-veined cheese produced on an industrial scale from raw or pasteurised cow and/or goat milk. The aim of this work was to evaluate the impact of pasteurisation on the microbiological, physicochemical and sensory characteristics of cheese. Cheeses made with raw milk showed higher counts of lactobacilli and enterococci, as well as lower values of pH and D-lactic acid and salt/moisture ratio. Cheeses made with pasteurised milk showed greater extent of proteolysis, higher concentration of free amino acids and lower concentration of biogenic amines. Pasteurisation produced more elastic and harder cheeses, but with a lower sensory profile.

Keywords Blue-veined cheese, Pasteurised milk, Proteolysis, Biogenic amines, Textural characteristics, Sensory properties.

INTRODUCTION

Valdeón cheese is a blue-veined variety manufactured in the municipal region of Posada de Valdeón, located in a National Park denominated Picos de Europa (León, Spain). The authenticity of this cheese is guaranteed since 2003 by a Protected Geographical Indication (PGI) (Commission Regulation (CE) 2004). Traditionally, this cheese was made from raw cow milk and/or goat milk during spring and summer. However, nowadays, the artisanal Valdeón cheesemaking has been replaced by industrial processes that use raw or pasteurised milk, producing the cheese throughout all the year. Consequently, industrial Valdeón cheeses produced with raw and pasteurised milk share general characteristics but differ to a greater or lesser extent, not only in their hygienic-sanitary quality but also in their composition and sensory properties. It is known that milk pasteurisation impacts on chemical changes during cheese maturation, and finally, on sensory characteristics (flavour and texture) (Licitra *et al*. 2019).

Proteolysis is the most complex and perhaps the most important biochemical event during the maturation of cheeses, being particularly important in blue-veined cheeses (Fox *et al*. 2017). Several studies have reported extensive proteolysis in blue cheese compared with other varieties (Gobbetti *et al*. 1997; Wolf *et al*. 2011; Mane *et al*. 2019). In fact, the pH 4.6-soluble nitrogen, as a percentage of total nitrogen, in some blue cheeses increases considerably during maturation reaching values of 30%, 45% or 55% in cheeses such as Danablu, Gorgonzola and Stilton, respectively. A wide range of enzymes from milk, chymosin, lactic starter and, in particular, mould culture (*Penicillium roqueforti*) contribute to the proteolysis and lipolysis of blue cheese, determining texture and flavour (Cantor *et al*. 2019; Caron *et al*. 2021). Proteolysis indirectly affects mouthfeel and flavour release during chewing. However, it also contributes to the off-flavour (e.g. bitterness) of cheese, through the formation of peptides and free amino acids, which may also serve as substrates for secondary catabolic changes (Sousa *et al*. 2001; Ganesan and Weimer 2017). Moreover, the decarboxylation of some amino acids in cheese leads to the formation of biogenic amines (BA), nonvolatile

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amines with important physiological effects in humans (Gaya *et al*. 2005). Many factors contribute to the presence and accumulation of BA, such as availability of free amino acids, pH, raw, salt-in-moisture level, temperature, redox potential bacterial density and, primarily, the presence of microorganisms (Schirone *et al*. 2012; Pleva *et al*. 2014; Kandasamy *et al*. 2021). Under normal conditions, exogenous BA ingested with food are rapidly detoxified by the action of amine oxidases. However, if the detoxification process is disturbed or the BA concentration in food is very high, BA becomes toxic metabolites responsible of serious human health problems (Linares *et al*. 2012). Thus, knowledge of BA levels in cheese is necessary to assess the health risks associated with the consumption of these products. Furthermore, they could be useful as indicators of the hygienic quality and manufacturing conditions used in cheese production.

It should be noted that previous studies have only been carried out on artisanal Valdeón cheese made with raw milk, prior to the industrialisation process (López-Díaz *et al*. 1995). Consequently, the aim of this work was to investigate and compare the microbiological, physicochemical and proteolytic parameters, especially the contents of free amino acids and BA, of Valdeón industrial cheeses made from raw and pasteurised milk, and their impact on the rheological, textural, colour and sensory characteristics of the cheeses at the end of the maturation.

MATERIALS AND METHODS

Materials

Twelve batches of Valdeón cheese were produced following the method described by Diezhandino *et al*. (2015). All were made from a mixture of cow and goat milk (90% and 10%, respectively). Eight of the batches were made from pasteurised milk using a commercial LAB mesophilic starter culture (FD-DVS CHN-19, Chr Hansen SL, Madrid, Spain) and a liquid spores suspension (1.6×10^8 spores/mL) of *P. roqueforti* (Biostar, Toledo, Spain). The other four batches were made from raw milk using only the liquid spore suspension of *P. roqueforti*. Cheeses were matured for 2 months (usual consumption date) in a drying room at 10°C and 90% relative humidity. The whole cheese is cylindrical in shape, with a maximum height of 15 cm, a diameter of 25 cm and a weight of approximately 2.4 kg. The paste is ivory-white in colour, evolving towards a cream colour, with a shiny intensity, smooth texture, with numerous homogeneously distributed, irregular hollows of variable size and blue-greenish colour. Its rind is natural, thin, soft and yellowish in colour and with greyish tones. Two whole cheeses were taken from each batch at 60 days of maturation for analysis. All measurements were performed in duplicate or triplicate.

Microbiological analysis

Microbiological analyses were performed on Plate Count Agar (PCA) (Oxoid, Unipath Ltd., Basingstoke, UK) after incubation at 30°C for 48 h and 7°C for 10 days for aerobic mesophilic and psychrotrophic bacteria counts, respectively, on M17 agar (Biokar, Beauvais, France) after incubation at 30°C for 18–24 h for presumptive lactococci counts, on MSE agar (Biokar) after incubation at 22°C for 4 days for presumptive *Leuconostoc* counts, on ROGOSA agar (Oxoid) after incubation at 30°C for 5 days for lactobacilli counts, on Mannitol Salt Agar (MSA) (Oxoid) after incubation at 30°C for 48 h for *Micrococcaceae* counts, on Kanamycin Aesculin Azide (KAA) agar (Oxoid) after incubation at 37°C for 18–24 h for enterococci counts and on Violet Red Bile Glucose Agar (VRBGA) (Oxoid) after incubation at 37°C for 18–24 h for *Enterobacteriaceae* counts, and finally, moulds and yeasts counts were performed on Oxytetracycline-Glucose-Yeast Extract (OGYE) agar (Oxoid) after incubation at 22°C for 5 days. On standard PCA, ROGOSA agar, KAA agar, VRBGA and OGYEA 1 mL volumes of each dilution were inoculated in duplicate. After solidification, ROGOSA agar and VRBGA plates were covered with a layer of the same medium. On M17 agar, MSE agar and MSA agar, 0.1 mL volumes of each dilution were surface plated. Plates with 30–300 colonies (15–150 for *Enterobacteriaceae*) were counted, and the counts were expressed as log cfu/g.

Physico-chemical analysis

Total solids (TS), protein and fat contents were determined following FIL-IDF standard 004 (2004), standard 20-1 (2001) and standard 221 (2008), respectively. NaCl content and pH were determined according to AOAC standard 935.43 (1990) and 14.022 (1980), respectively. D-lactic acid and L-lactic acid contents were determined using a Boehringer Mannheim enzymatic kit (R-biopharm, Roche, Germany). Water activity (aw) was determined using an Aqualab Dew Point Analyzer CX-2 (Decagon Devices Inc., Pullman, WA, USA).

Analysis of proteolytic parameters

At the end of the maturation, pH 4.6-soluble nitrogen (pH 4.6-SN), 12% trichloroacetic acid-soluble nitrogen (TCA-SN) and 5% phosphotungstic acid-soluble nitrogen (PTA-SN) were determined following FIL-IDF standard 224 (2011), using a preparation method described previously by Kuchroo and Fox (1982). The pH 4.6 insoluble fractions of the cheeses were analysed by urea-polyacrylamide gel electrophoresis (Shalabi and Fox 1987). Then, the different fractions were quantified using the gel analysis software TotalLab 1D, nonlinear Dynamix (Newcastle upon Tyne, UK). The pH 4.6 SN fractions of the cheeses were analysed by reverse-phase ultra-performance liquid chromatography (RP-UPLC) (Sousa *et al*. 2001). Elution was monitored at

214 nm and a mobile phase of two solvents: A, 0.1% (v/v) formic acid (sequencing grade; Sigma, St Louis, MO, USA) in deionised water (Milli Q System; Waters Corp., Barcelona, Spain) and B, 0.1% (v/v) formic acid in acetonitrile (HPLC grade; Lab-scan Ltd., Dublin, Ireland) were used. The chromatographic profiles were processed according to previously described method (Piraino *et al*. 2004). Finally, plasmin activity was performed using a modification of method of Richardson and Pearce (1981), using N-succinyl-L-Ala-L-Phe-L-Lys 7-amido-4-methyl-coumarin (AMC) as substrate. Plasmin activity was expressed as plasmin units per g cheese (1 unit was defined as the activity necessary to release 1 nmol AMC per min under standard assay conditions).

Amino acid analysis

Separation, identification and quantification of free amino acids were carried out by reverse-phase high-performance liquid chromatography (RPHPLC) (Alonso 1994). Chromatographic systems consisted of an HPLC Waters Alliance (Milford, Massachusetts, USA), equipped with a Waters 2695 separation module. The separation of amino acids was carried out using a C18 Brisa LC2 column (Teknokroma, Barcelona, Spain) (5 µm particle size, 250 mm × 4.6 mm I.D.) thermostated at 50°C ± 1. The detection was carried out by Waters 2487 (Milford, MA, EEUU) equipment at 254 nm. The volume injected was 20 µL. Free amino acids were separated by using a linear elution gradient with mobile phases A (sodium acetate trihydrate:acetonitrile, 94:6) and B (acetonitrile:water, 60:40).

Biogenic amines analysis

Extraction, derivation, separation, identification, and quantification of BA (tyramine, putrescine, cadaverine, spermine, tryptamine, phenylethylamine, histamine and spermidine) were carried out following previous methodology (Combarros-Fuertes *et al*. 2016). The chromatographic system consisted of an HPLC Waters Alliance (Milford, Massachusetts, USA), equipped with a Waters 2695 separation module connected to a Waters 2996 photodiode array detector. The separation of BA was carried out using a Waters Atlantis dC18 column (5 µm particle size, 150 mm × 4.6 mm I.D.) equipped with a Waters Atlantis dC18 guard-column (5 µm particle size, 20 mm × 4.6 mm I.D.). The volume injected was 20 µL. Biogenic amines were separated by using a linear elution gradient with mobile phases A (ammonium acetate 0.1 M) and B (acetonitrile, HPLC quality). The temperature of the column was set at 40°C ± 5°C. Peaks were detected at 254 nm, and the computer package used was Empower Version 2 by Waters™.

Rheological and texture profile analysis

Dynamic oscillatory analysis was performed using an AR2000ex Rheometer (TA Instruments Company, New Castle, DE, USA). Storage modulus (G'), loss modulus (G''), phase angle tangent (tan δ) and shear modulus (G^*) were determined under frequency rate from 0.06 to 628.30 rad/s in a linear viscoelastic region (previously determined taking amplitude strain sweep tests at a constant angular frequency of 6.28 rad/s). All measurements were conducted at 20°C. Using a steel wire, disc of 5 mm in height and 40 mm in diameter was removed from each cheese by triplicate. The force applied was uniformly set at 1 N for all samples. Data integration was carried out using the software incorporated into the equipment, namely Rheology Advantage 130 Data Analysis, version 5.7 (New Castle, DE, USA).

Texture profile analysis (TPA) was carried out in accordance with the methodology described by Bourne (1982). Texture parameters were determined using a TA-XT2 Texture Analyzer (Stable Micro System, Godalming, Surrey, UK). The test was carried out by compressing the samples to 80% using two compression cycles at a constant rate of 0.5 mm/s and at room temperature (20 ± 2°C). It was used a plate–plate sensor system with a stainless SMS P/75 probe.

Colour analysis

Colour was recorded using a Spectrophotometer CM-700 d (Konica Minolta, Osaka, Japan). The L^* (corresponding to dark/light), a^* (red/green) and b^* (yellow/blue) were determined according to CIELab. The MAV (measurement/illumination area) and MAV mask pattern were read with 8 mm diameter glass. It was used an illuminant D65 and an illumination angle of 10°. Each colour test was performed on the internal surface cheese by duplicate, and 12 measurements were carried out at different points of the surface uniformly selected to be representative of the different areas of the cheese. The results were analysed using a Color Data software CM-S100w SpectraMagic TM NX v. 1.9, Pro USB (Konica Minolta).

Sensory analysis

Sensory analysis was determined using a panel of 20 trained tasters at Food Hygiene and Technology Department of the University of León. Panellists were trained and acquainted with a defined vocabulary to describe the sensory parameters of blue cheese following the ISO 8586 (2012). A descriptive test was conducted, and twenty sensory attributes were scored, five for odour, seven for taste, five for texture and three for appearance, on a scale of 1 to 7 (Diezhandino *et al*. 2016). Finally, overall impression of each cheese was given a score on a scale from 1 to 10.

Statistics

An ANOVA/MANOVA analysis was used to compare means with a significant difference, setting a confidence interval of 95%. Statistical correlations were performed by means of Pearson's correlation coefficient. Both analyses

were carried out using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA). Principal component analysis (PCA) was performed by standardising the variables to zero mean and using a covariance matrix. Statistical analysis was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).

RESULTS

Cheese microbiota counts

In general, microbial counts obtained in raw milk cheeses were higher than those obtained in batches made from pasteurised milk (Table 1), except for moulds and yeasts counts, which were significantly lower in cheeses made from raw milk ($P < 0.05$). Similarities can be observed in the presumptive *Leuconostoc* and *Lactococcus* counts. However, *Lactobacillus* counts were approximately 2 log units higher in raw milk cheeses than in pasteurised milk cheeses. *Enterobacteriaceae* counts were not detected in pasteurised milk cheeses due to heat treatment; however, counts were elevated in raw milk cheeses. *Enterococcus* counts were very low or even undetected in cheeses made from pasteurised milk.

Physico-chemical parameters

Significant differences ($P < 0.05$) were observed between cheeses made with pasteurised and raw milk in several parameters such as S/M ratio (7.87 versus 6.57), pH values (7.08 versus 6.57) and aw (0.937 versus 0.957) (Table 2). No lactose was detected in Valdeón raw milk cheeses, showing a significantly higher D-lactic acid content than in pasteurised milk cheeses. No significant differences were observed in protein, fat, NaCl and L-lactic acid contents.

Assessment of cheese proteolysis

The mean values obtained for the different nitrogen fractions, expressed as g 100 g^{-1} of total nitrogen, are shown in Table 3. The soluble nitrogen at pH 4.6 (pH 4.6-SN) showed significant differences depending on the cheese ($P < 0.001$), with the pH 4.6-SN content being higher in cheeses made with pasteurised milk. No significant differences were observed in the TCA-SN and PTA-SN content. However, TCA-SN content expressed as a percentage of pH 4.6-SN was higher in raw milk cheeses (91.22%) than in pasteurised milk cheeses (76.56%), indicating a greater depth of proteolysis in cheeses made with raw milk. Polypeptide-N showed significant differences ($P < 0.05$) between cheeses. When the polypeptide-N was expressed as percentage of pH 4.6-SN, it was much higher in pasteurised milk cheeses (23.43%) than in raw milk cheeses (8.78%), where the depth of proteolysis was greater. No significant differences were observed in peptide-N content. The differences observed in nitrogen fractions agreed with those observed in the casein degradation study (see Table 3). Cheeses made from pasteurised milk showed a higher degradation of αs1-CN, αs1-I-CN and β-CN, indicating a

Table 1 Microbiological counts (log cfu/g) in Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days). Values represent the mean ± standard deviation of the batches.

Counts of	Pasteurised milk cheeses (eight batches)	Raw milk cheeses (four batches)	Significant differences[a]
Aerobic mesophilic bacteria	7.49 ± 0.67	8.40 ± 0.78	NS
Aerobic psychrotrophic bacteria	7.18 ± 0.74	8.44 ± 0.00	NS
Presumptive *Leuconostc*	6.20 ± 0.91	6.36 ± 0.21	NS
Lactobacillus	5.30 ± 0.95	7.57 ± 0.46	*
Presumptive *Lactococcus*	5.45 ± 0.90	6.36 ± 0.48	NS
Micrococcaceae	3.82 ± 0.76	3.37 ± 0.32	NS
Enterococcus	2.42 ± 1.12	6.30 ± 0.78	*
Moulds and yeasts	8.59 ± 0.26	7.88 ± 0.10	*
Enterobacteriaceae	ND[b]	5.66 ± 0.23	***

[a]Last column shows the significant differences. NS, no significant differences; *$P < 0.05$; ***$P < 0.001$.
[b]ND, no detected.

Table 2 Physico-chemical characteristics of Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days). Values represent the mean ± standard deviation of the batches.

	Pasteurised milk cheeses (eight batches)	Raw milk cheeses (four batches)	Significant differences[a]
Total solids[b]	60.87 ± 2.79	58.49 ± 0.12	*
Protein[c]	33.93 ± 1.73	36.59 ± 0.85	NS
Fat[c]	55.00 ± 2.23	51.70 ± 0.91	NS
Lactose[c]	0.14 ± 0.02	ND[e]	*
D-lactic acid[c]	0.16 ± 0.03	0.39 ± 0.07	*
L-lactic acid[c]	0.49 ± 0.24	0.56 ± 0.12	NS
NaCl[c]	5.06 ± 0.35	6.19 ± 0.45	NS
Salt/moisture[d]	7.87 ± 0.07	6.57 ± 0.21	*
pH	7.08 ± 0.39	6.57 ± 0.06	*
aw	0.937 ± 0.01	0.957 ± 0.01	*

[a]Last column shows the significant differences. NS, no significant differences; *$P < 0.05$.
[b]Expressed as g 100 g^{-1} of cheese.
[c]Expressed as g 100 g^{-1} of total solids.
[d]Expressed as g of NaCl 100 g^{-1} of moisture.
[e]ND, no detected.

Table 3 Soluble nitrogenous components and pixel intensity (volume) of the band of the electrophoretic regions in the stained gels of caseins of Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days). Values represent the mean ± standard deviation of the batches.

	Pasteurised milk (eight cheeses batches)	Raw milk (four cheeses batches)	Significant differences[a]
pH 4.6-SN[b]	39.98 ± 0.78	33.72 ± 3.68	***
TCA-SN[b]	30.61 ± 0.87	30.76 ± 3.30	NS
PTA-SN[b]	13.65 ± 0.39	11.69 ± 0.49	NS
Polypeptide-N[b]	9.37 ± 1.65	2.96 ± 0.37	*
Peptide-N[b]	16.97 ± 0.48	19.60 ± 2.81	NS
γ_2-CN	525.88 ± 110.61	383.52 ± 34.80	NS
γ_1-CN	285.61 ± 84.30	273.51 ± 6.32	NS
γ_3-CN	510.59 ± 109.63	511.46 ± 45.85	NS
β-CN	469.72 ± 94.06	735.13 ± 47.58	**
αs_1-CN	266.30 ± 70.78	329.94 ± 85.25	NS
αs1-I-CN	317.13 ± 61.01	746.58 ± 100.01	**

[a]Last column shows the significant differences. NS, no significant differences; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[b]Expressed as g 100 g^{-1} of total nitrogen.

higher formation of high and medium molecular weight peptides.

Figure 1(a) shows the score plot obtained from the principal component analysis of the peptide profile of soluble extracts at pH 4.6 of cheeses made from pasteurised and raw milk, obtained by RP-UPLC. The variables obtained were transformed into classes (peptides or groups of peptides) using a logistic weighting function (60 classes). Principal components (PC) accounted for 76.7% of the total variation in the peptide profile of the cheeses (49.6% PC1 and 27.1% PC2). The largest differences between samples were observed in the retention time from 1.7 to 3.3 min. Vector loadings showed that seven main classes explained most of the observed differences (Figure 1b). The numbers in the graph signify the status of the retention time classes in the total of 60. Cheeses made from raw milk (to the right of PC1) were characterised as belonging to classes 6, 30 and 31 (1.7 and 3.2 min), while cheeses made from pasteurised milk were to the left of PC1 and were included in classes 20, 28, 29 and 32 (2.4, 3.0, 3.1 and 3.3 min, respectively).

Free amino acid content

Figure 2 shows the free amino acid content found in Valdeón cheese made from pasteurised and raw milk at 60 days of maturation. The free amino acid content of 60-day aged cheeses made from pasteurised milk and raw milk was 1961.66 and 1788.75 mg 100 g^{-1} of total solids, respectively. The predominant free amino acids were Glu, Ala, Pro, Tyr, Lys, Asp, Leu, Pro and Phe. Several amino acids (Asn, Ser, Gly, Arg+Tau, Gaba+Thr, Trp, Orn, Asp, Tyr and Lys) showed higher concentrations in cheeses made from pasteurised milk, while the content of other amino acids (Cit, Eta, Met, Cys, Glu and Ala) was higher in cheeses made from raw milk.

Biogenic amines content

Figure 3 shows the BA content of Valdeón cheese made from pasteurised and raw milk after 60 days of maturation. The total BA concentration of the cheeses made from raw milk was almost twice that of those made from pasteurised milk (1701.75 and 1060.95 mg/kg of cheese, respectively). Putrescine, cadaverine and tyramine showed significant differences ($P < 0.05$) between cheeses. Histamine and spermidine contents were low in both raw milk and pasteurised milk cheeses. Spermine content was higher in pasteurised milk cheeses. The large difference observed in tyramine content between cheeses was remarkable, with cheeses made from pasteurised milk showing content twice lower than cheeses made from raw milk (244.45 versus 565.24 mg/kg of cheese, respectively). In addition to tyramine, the contents of tryptamine, histamine, cadaverine and putrescine were also higher in cheeses made from raw milk.

Rheology, texture profile and colour analysis

The main values obtained in the dynamic oscillatory test for Valdeón cheese are shown in Table 4. Both G' and G'' were significantly higher ($P < 0.05$) in cheeses made with pasteurised milk, characterised by a more elastic structure and a greater capacity to resist deformation. Cheeses made with pasteurised milk had lower water content and higher pH values and these two parameters lead to a reduction of tan δ. On the contrary, cheeses made with raw milk showed a higher depth of proteolysis, which results in an increase of this parameter. However, no significant differences in tan δ values were observed. Finally, cheeses made with pasteurised milk showed significantly higher G^* values ($P < 0.05$) due to the lower water content resulting in a much firmer cheese matrix (firmness effect).

Table 4 shows the results obtained in the texture profile analysis (TPA) of Valdeón cheese. TPA revealed significant differences ($P < 0.05$) in fracturability, hardness, cohesiveness and springiness between the cheeses. The higher values of fracturability and hardness observed in pasteurised milk cheeses coincided with the results obtained in the dynamic oscillatory test. In fact, a positive correlation was found between these two textural attributes and G^* values ($r = 0.94$ and $r = 0.92$, respectively) ($P < 0.05$). Cohesiveness and springiness values were significantly lower in pasteurised milk cheeses.

L^* values were significantly lower in the pasteurised milk cheeses (Table 5) due to the higher total solids content, with

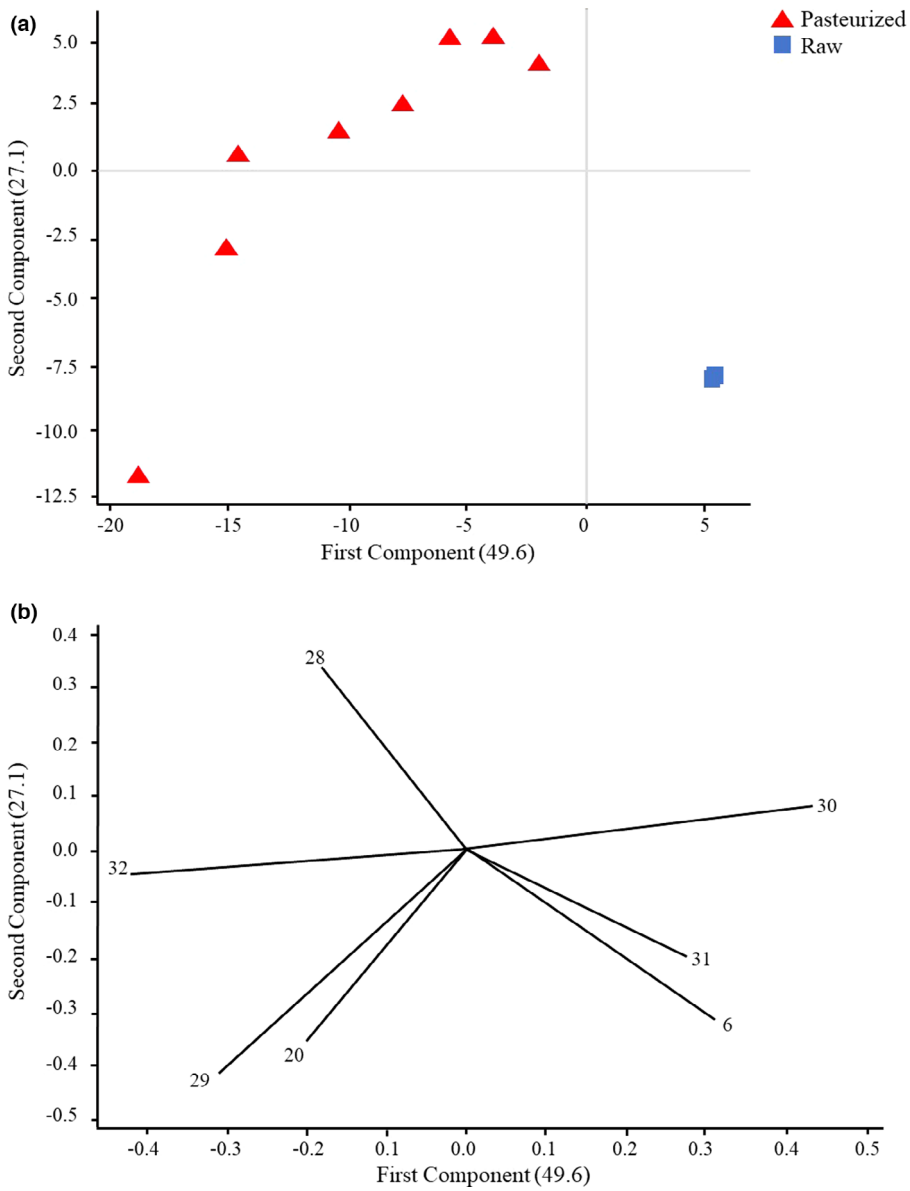

Figure 1 Score plot (a) and loading vectors (b) of variables with high loading obtained by principal component analysis of reverse-phase UPLC chromatograms of the pH 4.6-soluble extracts from Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days).

a negative correlation between L* values and total solids ($r = -0.99$) ($P < 0.01$). The a^* values were significantly lower ($P < 0.001$) in cheeses made with pasteurised milk due to the higher growth of *P. roqueforti*, which resulted in a higher green colour. Finally, b^* values were significantly higher ($P < 0.05$) in cheeses made with raw milk, showing a higher predominance of yellowish colour. A positive correlation was observed between blue-green colour of the veins and *moulds and yeasts* counts ($r = 0.88$) ($P < 0.05$).

Sensory analysis

Figure 4 shows the results obtained in the sensory analysis of Valdeón cheese. In terms of appearance, the score given to the blue-green colour of the veins was significantly higher ($P < 0.05$) in cheeses made from pasteurised milk due to the higher growth of *P. roqueforti* in these cheeses. Cheeses made with raw milk showed lower bitterness and astringency, as well as higher sweetness scores ($P < 0.05$). In fact, cheeses made with pasteurised milk showed 2.6 higher polypeptide-N values than those obtained in cheeses made with raw milk, while the latter had a higher hydrophilic/hydrophobic ratio. In terms of texture, cheeses made with pasteurised milk showed significantly higher firmness scores ($P < 0.05$), which are in agreement with the dynamic oscillatory and TPA results. In fact, a positive correlation was observed between these results and the G^* values obtained

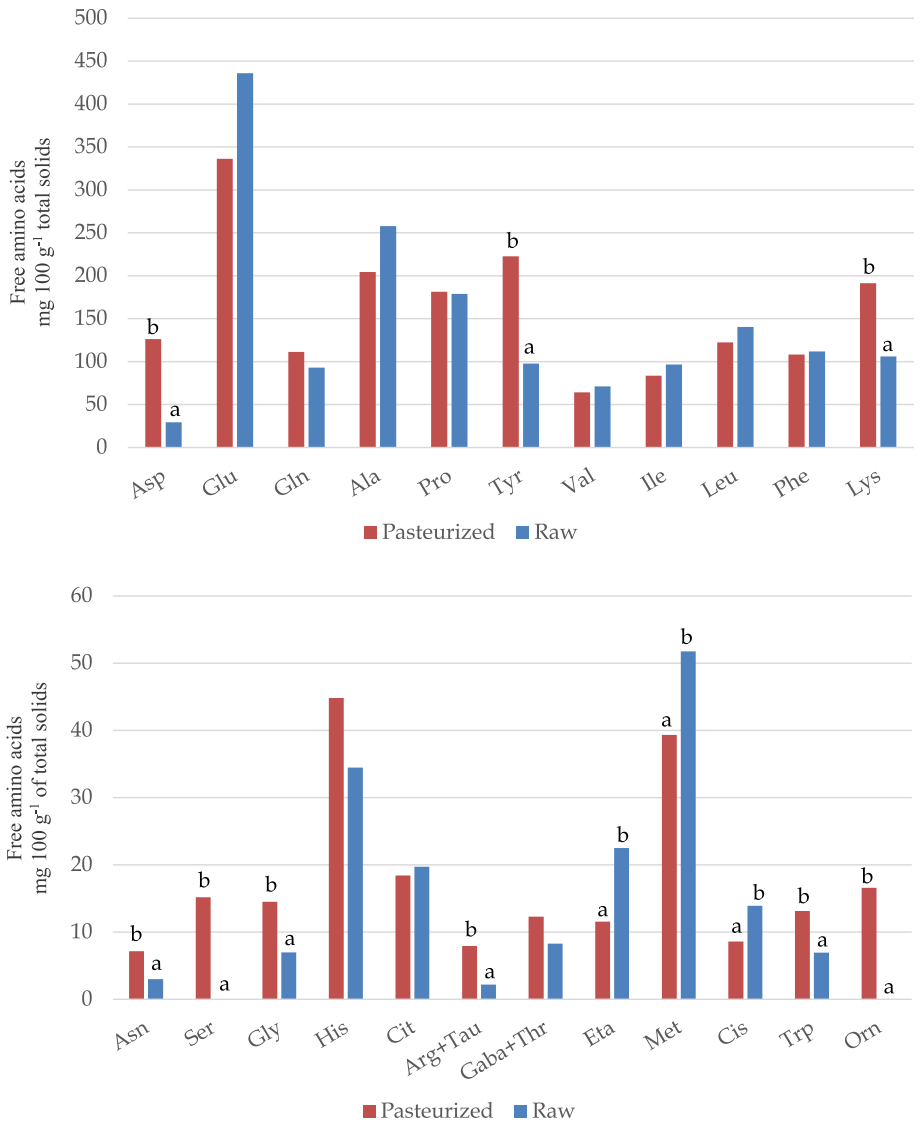

Figure 2 Free amino acid contents (mg 100 g^{-1} total solids) found in Valdeón cheese made from pasteurised (P) and raw milk (R) at 60 days of maturation. Means with different superscript differ significantly ($P < 0.05$).

in the dynamic oscillatory test ($r = 0.99$) ($P < 0.05$) and the hardness and fracturability values obtained in the TPA ($r = 0.96$ and $r = 0.99$, respectively) ($P < 0.05$). The negative correlation observed between hardness (TPA) and buttery values ($r = -0.97$) ($P < 0.05$) was very interesting, which coincided with the results reported by other authors (Adhikari *et al.* 2003; García *et al.* 2015).

DISCUSSION

A starter culture consisting of *Lactococcus* and *Leuconostoc* strains was incorporated during the production of pasteurised milk cheeses, which justifies the similar presumptive *Lactococcus* and *Leuconostoc* counts in raw and pasteurised milk cheeses. However, *Lactobacilli* are nonstarter lactic acid bacteria (NSLAB) and the population present in raw milk increases gradually throughout the maturation of the cheeses. Cheeses manufactured from pasteurised milk acquire an adventitia population of lactobacilli from the environment that multiplies, but whose counts are lower than expected for cheeses made from raw milk of the same maturation time. In cheeses made from raw milk, there is a microbial community from the milk that manages to survive until the end of maturation, when salt/moisture conditions are more restrictive (Yunita and Dodd 2018). Counts of Aerobic mesophilic bacteria, moulds and yeasts and *Lactobacillus* in cheeses made from raw milk were similar to those previously reported in Valdeón artisanal cheese, while counts on *Micrococcaceae* and presumptive *Leuconostoc* were lower (López-Díaz *et al.* 1995). *Enterobacteriaceae*

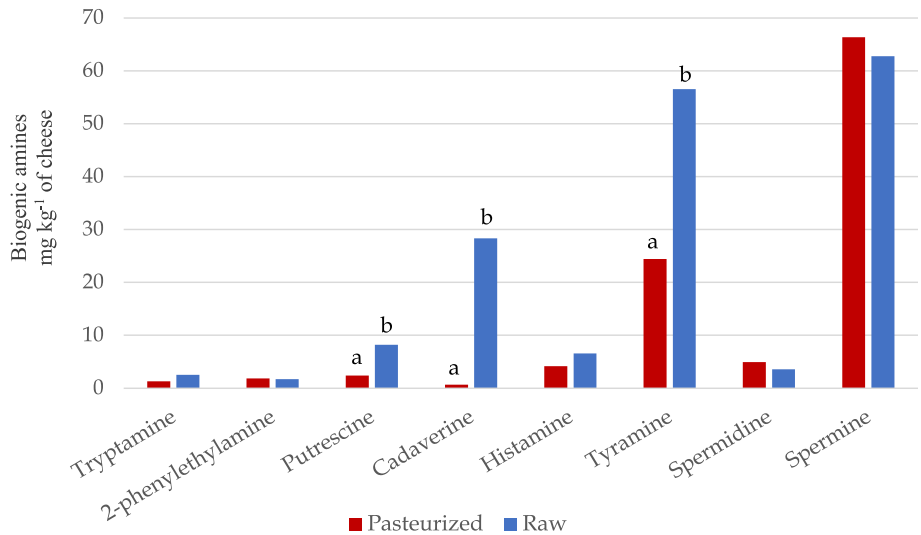

Figure 3 Biogenic amines contents (mg/kg of cheese) found in Valdeón cheese made from pasteurised (P) and raw milk (R) at 60 days of maturation. Means with different superscript differ significantly ($P < 0.05$).

Table 4 Rheological and texture profile analysis parameters of Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days). Values represent the mean ± standard deviation of the batches.

	Pasteurised milk cheeses (eight batches)	Raw milk cheeses (four batches)	Significant differences[a]
G' (MPa)	108.24 ± 36.90	43.88 ± 9.56	*
G''(MPa)	28.12 ± 9.30	11.86 ± 2.42	**
*G**(MPa)	111.87 ± 38.02	45.46 ± 9.86	*
Tan δ	0.26 ± 0.02	0.27 ± 0.01	NS
Fracturability (N)	5.51 ± 2.10	1.43 ± 0.01	*
Hardness (N)	52.36 ± 17.87	28.94 ± 4.69	*
Adhesiveness (N.s)	-8.79 ± 2.26	-8.53 ± 0.77	NS
Cohesiveness	0.10 ± 0.02	0.17 ± 0.06	*
Springiness	0.26 ± 0.03	0.35 ± 0.02	*
Gumminess	5.31 ± 1.96	4.95 ± 2.68	NS
Chewiness	1.42 ± 0.59	1.81 ± 1.06	NS

[a]Last column shows the significant differences. NS, no significant differences; *$P < 0.05$; **$P < 0.01$.

Table 5 Colour parameters of Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days). Values represent the mean ± standard deviation of the batches.

	Pasteurised milk cheeses (four batches)	Raw milk cheeses (four batches)	Significant differences[a]
*L**	71.39 ± 8.04	77.1 5 ± 7.34	**
*a**	-2.40 ± 0.77	−1.53 ± 1.12	***
*b**	11.21 ± 2.69	12.64 ± 3.02	*

[a]Last column shows the significant differences. NS, no significant differences; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

counts were comparable to those reported by other authors in similar varieties, such as Cabrales (Flórez and Mayo 2006; Flórez *et al*. 2006).

The moisture content of the cheese influences the maturation process and affects the weight loss of the cheese (Hay 2017). In general, aw values for blue cheeses are lower than those described for other cheese varieties, reaching values below 0.90 (Flórez *et al*. 2006). The decrease in aw values, experienced by cheeses during maturation, is mainly influenced by the presence of salt, moisture loss and the gradual hydrolysis of proteins to low molecular weight soluble compounds, with the high salt/moisture content of these varieties having the greatest influence on aw (Fernández-Salguero *et al*. 2004). The optimum aw for *P. roqueforti* growth is 0.998 and the minimum 0.840, with its adaptation phase increasing at aw values below 0.920 (Valík and Görner 1999). Therefore, aw values set in pasteurised and raw milk cheeses (0.937 and 0.957, respectively) could allow to *Penicillium roqueforti* to germinate rapidly and grow during cheesemaking and maturation, and, in turn, limit the growth of some pathogenic microorganisms such as *Staphylococcus aureus* and *Listeria monocytogenes* (Wemmenhov *et al*. 2021). The D-lactic acid content is associated with high lactobacilli counts in the cheese. *Lactobacilli* produce the conversion of L-lactic acid to the D-lactic acid, due to the action of racemases and also produce D-lactic acid from the

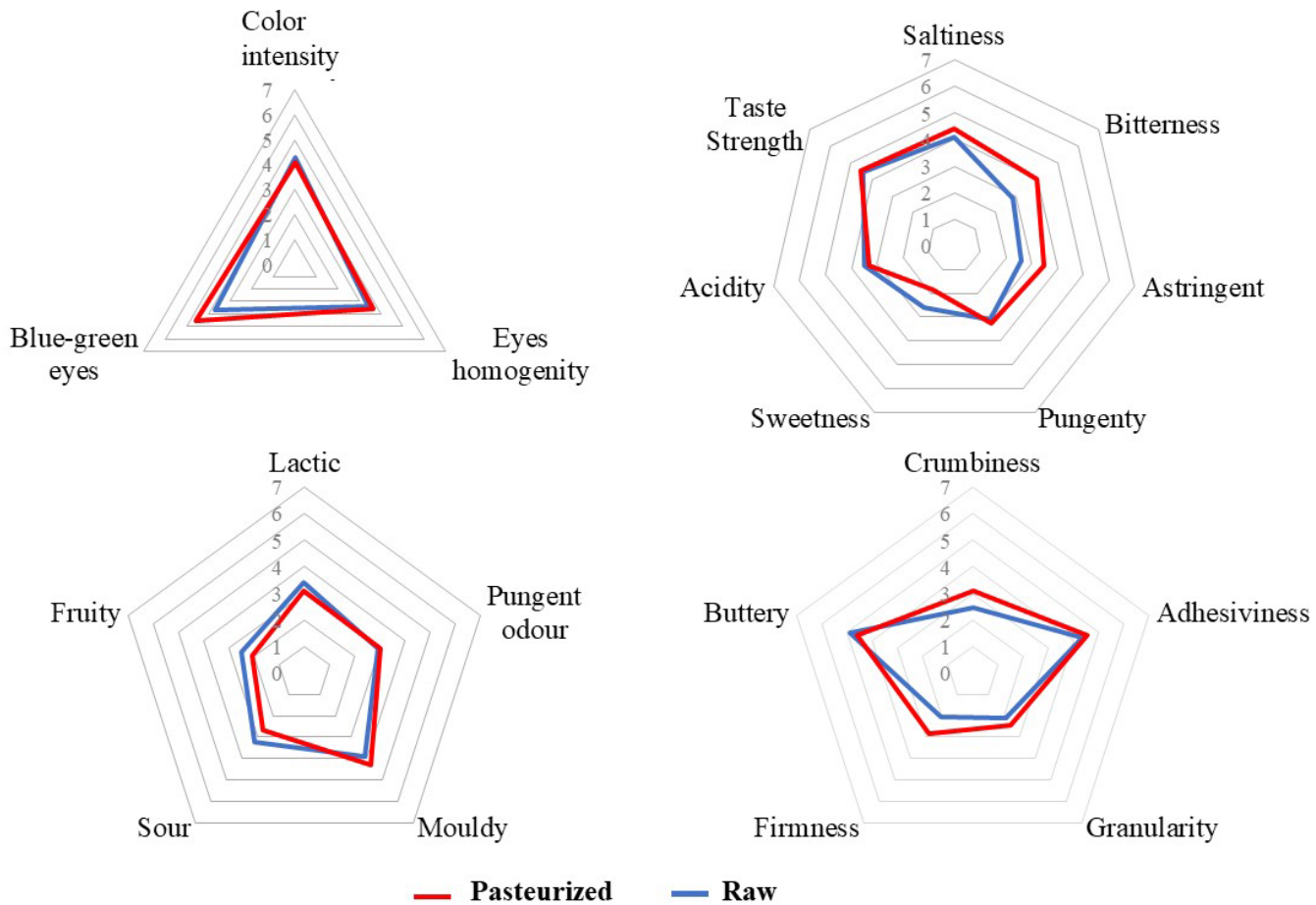

Figure 4 Sensory scores: (a) appearance, (b) taste, (c) odour and (d) texture of Valdeón cheese made from pasteurised and raw milk at the end of maturation (60 days).

fermentation of galactose. The lower pH values of the raw milk cheeses could be associated with a higher count of NSLAB bacteria, such as lactobacilli, and lower mould growth and thus lower lactic acid catabolism (Hayaloglu *et al*. 2008). In cheeses made with pasteurised milk, mould growth was higher, resulting in higher lactic acid metabolism.

Results obtained for the soluble nitrogen at pH 4.6 (pH 4.6-SN) indicated a higher extent of proteolysis in pasteurised milk cheeses, in agreement with the results obtained for casein degradation (as will be discussed below). This could explain their lower aw values, since one of the effects of proteolysis is the breaking of peptide bonds releasing two new charged groups (NH_3^+/COO^-) that compete for water, thus reducing the aw (O'Mahony *et al*. 2005). Similarly, in Danish blue, pH 4.6-SN increased by more than three times, indicating the extent of proteolysis (Mane *et al*. 2019). The PTA-SN and TCA-SN contents are in agreement with those reported in similar varieties where higher secondary proteolysis was observed in cheeses made with raw milk as a consequence of higher aminopeptidase activity from the diversity of microbiota present in raw milk (Licitra *et al*. 2019). The variety of peptides and amino acids is related to the diversity of microbiota, especially the NSLAB group (Bottari *et al*. 2020).

Differences were also observed in the study of casein degradation, which was higher in cheeses made with pasteurised milk. Primary proteolysis is mainly associated with the coagulant enzyme involved in the hydrolysis of αs1-CN (Fox *et al*. 2017). Plasmin is known to hydrolyse β-CN to γ-CN and pasteurisation increases plasmin activity due to inactivation of plasmin inhibitors and/or denaturation of plasminogen activator inhibitors (Bastian and Brown 1996). In fact, plasmin activity was higher in pasteurised milk cheeses (3.30 plasmin units per g) than in raw milk cheeses (1.23 plasmin units per g). The lower acidification observed in pasteurised milk cheeses could favour the higher activity of plasmin, as the optimum pH of this enzyme is around 7.5. On the other hand, the action of proteases released by *P. roqueforti*, which have been described as the main proteolytic agent in blue-veined cheeses, must be considered. *P. roqueforti* proteinases act by hydrolysing specific peptide bonds in αs1-CN and β-CN (Mane *et al*. 2019). Therefore, the higher growth of *P. roqueforti* in pasteurised milk

cheeses could also explain the higher degradation of αs1- and β-CN. Efficient proteolysis influences the formation of aromatic compounds (Caron *et al*. 2021).

Cheeses made from raw milk showed a slightly higher hydrophilic/hydrophobic ratio than those made from pasteurised milk, as they were characterised in class 6 corresponding to the more hydrophilic peptides, which elute at a lower concentration of acetonitrile. Remarkable proteolysis that occurs causes the degradation of hydrophobic peptides and the release of low molecular weight peptides and amino acids.

The use of *P. roqueforti* as a secondary starter culture during cheesemaking standardised the production process and led to an enhanced proteolysis of the cheeses.

Although there were no significant differences in TCA-SN and PTA-SN values between cheese made from pasteurised and raw milk, differences in the amino acid profile were observed.

Free amino acid content was similar to those obtained in other studies for some amino acids, such as Gly and His, but differed in the content of the main amino acids (Mane *et al*. 2019). It highlighted the differences found in Asn since its concentration could be used as pasteurisation indicator because the heat treatment causes inactivation of asparaginase, allowing it to accumulate in the cheese (Frau *et al*. 1997). Orn and Ser were not detected in cheeses elaborated from raw milk. It should be noted that contaminating microorganisms as *Enterobacteriaceae* produce putrescine from Orn decarboxylation (Linares *et al*. 2012). In fact, raw milk cheeses showed higher putrescine content. Gaba is produced by glutamic acid decarboxylation by microorganisms, especially *Lactobacillus* strains, as a mechanism of intracellular pH regulation under acidic conditions (Ardö 2006). Several authors have studied the importance of Gaba in terms of its impact on health (Diana *et al*. 2014). The predominant free amino acids are of particular interest in cheese because of their relationship to distinct flavours: lysine to spicy, proline to sweet and glutamic acid to salty-umami (Kabelová *et al*. 2009).

Pasteurisation reduces the microbiota present in milk, including BA-producing microorganisms. Therefore, cheeses made with pasteurised milk tend to have lower BA concentrations (Linares *et al*. 2012). In addition, the cofactor for decarboxylase activity (pyridoxal phosphate) is heat sensitive, and so pasteurisation may also contribute to cheeses made with pasteurised milk having lower amine content values (Novella-Rodríguez *et al*. 2003). Results obtained for putrescine, cadaverine and tyramine were associated with the higher *Enterococcus* counts observed in cheeses made from raw milk. In fact, there was a significant positive correlation ($r = 0.79$) ($P < 0.01$) between tyramine and *Enterococcus* counts. A high concentration of tyramine constitutes a health risk and can be used as an indicator of hygienic conditions during manufacture. Although it is difficult to find a correlation between the presence of a high concentration of BA in cheese with an increase of a specific group of LAB, in certain cases this trait can be considered as characteristic of a particular genus, such as tyramine production by *Enterococcus* (Ladero *et al*. 2012; Calzada *et al*. 2013). The high level of tyramine in Pecorino cheese was related to the activity of heat-resistant *Enterococci* (common contaminants of raw milk) (Schirone *et al*. 2011). However, the role of NSLAB, such as *Lactobacillus*, especially *Levilactobacillus plantarum*, which have also been associated with high tyramine concentration in cheese, cannot be underestimated (Schirone *et al*. 2011). *Enterobacteriaceae* would be associated with the formation of cadaverine, putrescine and histamine (Marino *et al*. 2003). In fact, in Valdeón cheese, *Enterobacteriaceae* counts were significantly higher in raw milk cheeses than in pasteurised milk cheeses where no counts were detected. A significant positive correlation was found in *Enterobacteriaceae* counts with respect to the levels of cadaverine ($r = 0.99$) ($P < 0.001$), putrescine ($r = 0.99$) ($P < 0.001$) and histamine ($r = 0.83$) ($P < 0.01$). Histamine and tyramine were predominant in other mould ripened blue cheeses, at values below the maximum intake level (Reinholds *et al*. 2020).

The differences in fracturability, hardness, cohesiveness and elasticity observed between the cheeses could be related to the lower water content and higher pH values in cheeses made with pasteurised milk. When the water content in the protein matrix decreases, its consistency increases. Moreover, the pH of the cheese directly affects the texture of the curd by influencing the solubility of the caseins. In fact, a positive correlation has been described between the pH values inside cheeses and their hardness and fracturability (Fresno and Álvarez 2012). The lower cohesiveness and springiness values observed in pasteurised milk cheeses are possibly related to their higher fat and lower water content. The decrease in cohesiveness could be associated with the loss of elastic structural elements and the decrease in water content for protein solvation. In fact, several authors observed a negative correlation between fat content and cohesiveness, while others observed a decrease in springiness values with increasing fat content (Fresno and Álvarez 2012).

Cheeses made with pasteurised milk were characterised by a lower brightness, as well as a greater development of blue-greenish and less yellowish colorations. These results agree with those reported by Buffa *et al*. (2001) in goat cheese. These colour characteristics were related to a more uniform development and proliferation of moulds in the mass of cheeses made from pasteurised milk.

The differences observed in bitterness could be related to the higher extent of primary proteolysis in cheeses made with pasteurised milk, which results in a greater accumulation of small molecular size hydrophobic peptides, responsible for bitterness. The higher scores ($P < 0.05$) observed for

sour odour in cheeses made from raw milk was due to the greater accumulation of short-chain volatile acids released in the oxidative deamination of amino acids as these cheeses underwent a deeper proteolysis. Results obtained showed a good correlation between instrumental and sensory analysis. Finally, although no significant differences were observed in the overall impression, cheeses made from raw milk showed slightly higher scores (7.56) than cheeses made from pasteurised milk (6.83) due to a more balanced flavour, a more complex aromatic profile and low values for firmness, bitterness, astringency and crumbliness. In fact, 'crumbly texture' is negatively correlated with moisture and aw, and values for these parameters were higher in raw milk cheeses. Some authors suggest that consumer groups could be established according to preference profiles based on taste intensity (Bord *et al*. 2017).

CONCLUSION

Pasteurisation significantly reduced the contaminating microbiota as well as the total content of biogenic amines especially tyramine concentration, which resulted in cheeses with a lower health risk for the consumer and better development of the *P. roqueforti* mould. Both the cheeses made with raw and pasteurised milk presented physicochemical characteristics that were within the range established by the Regulatory Board of the Protected Geographical Indication of Valdeón cheese. Pasteurised cheeses showed greater extension in the degree of proteolysis but with a lower quality sensory profile. Cheeses made from raw milk showed overall scores slightly higher due to a more complex aromatic profile, a lower hardness and fracturability, as well as a lower degree of bitterness and astringency and a higher creaminess.

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AUTHOR CONTRIBUTIONS

Isabel Diezhandino: Data curation; Formal analysis; Investigation; Methodology; Writing – original draft. **Domingo Fernández:** Data curation; Formal analysis; Methodology. **Patricia Combarros-Fuertes:** Methodology. **Erica Renes:** Methodology. **José María Fresno:** Conceptualization; Writing – review & editing. **María Eugenia Tornadijo:** Conceptualization; Writing – review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in 'BULERIA' at http://doi.org/10.18002/10612/5449, reference number [1088].

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