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Autochthonous cultures to improve the quality of PGI Castellano cheese: impact on proteolysis, microstructure and texture during ripening

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ABSTRACT

The aim of this research was to find out the effect of different combinations of starter and non-starter cultures on the proteolysis of Castellano cheese during ripening. Four cheese batches were prepared, each containing autochthonous lactobacilli and or *Leuconostoc*, and were compared with each other and with a control batch, that used only a commercial starter. To achieve this, nitrogen fractions (pH 4.4-soluble nitrogen and 12% trichloroacetic acid soluble nitrogen, polypeptide nitrogen and casein nitrogen), levels of free amino acids and biogenic amines were assessed. Texture and microstructure of cheeses were also evaluated. Significant differences in nitrogen fractions were observed between batches at different stages of ripening. The free amino acid content increased throughout the cheese ripening process, with a more significant increase occurring after the first 30 days. Cheeses containing non-starter lactic acid bacteria exhibited the highest values at the end of the ripening period. Among the main amino acids, GABA was particularly abundant, especially in three of the cheese batches at the end of ripening. The autochthonous lactic acid bacteria were previously selected as non-producers of biogenic amines and this resulted in the absence of these compounds in the cheeses. Analysis of the microstructure of the cheese reflected the impact of proteolysis. Additionally, the texture profile analysis demonstrated that the cheese's hardness intensified as the ripening period progressed. The inclusion of autochthonous non-starter lactic acid bacteria in Castellano cheese production accelerated the proteolysis process, increasing significantly the free amino acids levels and improving the sensory quality of the cheeses.

Key words: Sheep's cheese; Lactic acid bacteria; Nitrogen fractions; Free amino acids; Microstructure; Texture profile analysis.

1. Introduction

Castellano cheese is an uncooked, enzymatically coagulated cheese, made from sheep's milk from farms in Castilla y León (Spain). The authenticity of this cheese will be guaranteed from 2020 by a Protected Geographical Indication (PGI) (European Commission, 2020). Castellano cheese is currently produced on a semi-industrial and industrial scale from raw milk and preferably pasteurised milk. In 2023, three million units of this variety were produced using 16 million liters of milk at an average price of €18 per kilogram of cheese. Currently, exports account for 6% of the market, but the medium-term forecast predicts a rise to 40%, with the USA being the primary foreign market. The PGI Castellano cheese covers 23 cheese factories, 16 cooperatives, and 950 livestock farms, according to information from the Federación Castellano-Leonesa de Industrias Lácteas, recognised PGI Castellano Cheese Board (BOCyL, 2021). The production process is as follows. Castellano PGI cheese can be made using raw or pasteurised sheep milk. The milk is heated to a temperature between 27 and 37 °C. Lactic starters and additives authorised by current legislation can be added at this point. Natural rennet or other coagulating enzymes are then added, and the mixture is left to coagulate for between 15 and 55 min. The curd is then cut into grains the size of rice grains or hazelnuts if more whey is to be retained. The curd is then gently stirred while gradually increasing the temperature, being careful not to exceed 39 °C. Finally, the curd is placed into cylindrical moulds and salted either wet or dry. When immersing in brine, the maximum duration should not exceed 72 h. The cheeses are ripened in chambers with a relative humidity ranging from 65% to 95% and a temperature between 3 and 18 °C. Necessary turning and cleaning techniques are applied until the cheese reaches its specific characteristics. If the weight of the cheese is less than 1.5 kg, it must mature for a minimum of 30 days. For weights exceeding 1.5 kg, the minimum ripening period is 60 days (European Commission, 2020).

During the ripening process, proteolysis is considered the most important biochemical process that takes place in hard and semi-hard cheeses, such as PGI Castellano, such as it promotes a series of reactions that determine the sensory characteristics of the cheese (Gaglio et al., 2020). During this process, the protein matrix of the cheese is broken down because of the action of various proteinases and peptidases from different sources: milk enzymes, coagulation enzymes, microbial enzymes from the starter culture and secondary microbiota, and other exogenous enzymes (Khattab et al., 2019). Microstructure plays a crucial role in determining cheese quality, as the arrangement of components at the microscopic level affects the texture, sensory perception, and overall quality of the cheese (Ong et al., 2022). In addition, it contributes to an increase in the content of free amino acids (FAA) and other compounds that serve as substrates for subsequent biochemical reactions (Araújo-Rodrigues et al., 2023). However, the release of some FAA, produced during cheese ripening, can act as precursors of biogenic amines (BA), which can accumulate in cheese and cause health problems in susceptible consumers (Renes et al., 2021). In fact, long-ripened cheeses have often been associated with BA poisoning, mainly due to the accumulation of tyramine (Ladero et al., 2010).

The use of pasteurised milk in cheese production has numerous technological and health benefits (Ruvalcaba-Gómez et al., 2022). However, the application of the heat treatment to the milk leads to the destruction of the native microbiota, which makes the use of starter cultures indispensable. Lactic acid bacteria (LAB) are responsible for a large part of the characteristics of the cheese (Araújo-Rodrigues et al., 2023). In general,

commercial starter LAB (SLAB) are composed of a few strains of LAB, mainly lactococci, responsible for lactic acid fermentation. However, the composition of these commercial cultures differs from the diversity of the native cheese microbiota, which is mainly composed of enterococci and lactobacilli and, to a lesser extent, *Leuconostoc* spp. and thermophilic LAB (De Pasquale et al., 2019). These LAB, called non-starter LAB (NSLAB), generally proliferate, and remain constant during cheese ripening, playing a key role in the secondary proteolysis of cheese through the release of low molecular weight peptides and FAA, which results in the formation of flavouring compounds (Blaya et al., 2018).

Previous research has demonstrated that the autochthonous lactic microbiota, mainly NSLAB, is primarily responsible for the variations in characteristics between cheeses made from raw milk and those made from pasteurised milk to which SLAB have been added. These differences manifest as an increase in the intensity of flavour and aroma in cheeses that retain the autochthonous microbiota, as well as a slight acceleration of proteolysis (Santos de Oliveira et al., 2023). Similarly, the relationship between the development of mesophilic lactobacilli and greater proteolytic activity has also been demonstrated (Gobbetti et al., 2015; Khattab et al., 2019). This set of proteolytic phenomena promotes changes in texture, causing the release of amino and carboxyl groups which contribute to the reduction in water activity and the increase in pH, facilitating the release of sapid compounds during chewing. The changes in the protein and lipid matrix that take place during ripening, due to proteolysis and lipolysis, will have an impact on the microstructure of the cheese (Milesi et al., 2009; Santos de Oliveira et al., 2023). Consequently, there has been a growing interest in the role of NSLAB and the selection and integration of autochthonous lactobacilli and *Leuconostoc* spp. in cheese production in recent years. This is particularly interesting for cheeses with quality designations like PGI Castellano cheese. Therefore, the objective of this research was to compare the impact of various autochthonous SLAB and NSLAB on the depth and extent of proteolysis during the ripening process of Castellano PGI cheese, as well as on its texture profile and microstructure.

2. Materials and methods

2.1. LAB strains, cheese manufacture and sampling

Five batches of Castellano cheese (one control batch and four experimental batches) were produced in duplicate (n=2) on a pilot scale using pasteurised sheep's milk. Ten cheeses were obtained from each batch, giving a total of 100 cheeses. Manufacturing was carried out in the pilot plant of Queserías Entrepinares (Valladolid, Spain) according to the procedure approved in the specifications of the PGI Castellano cheese (Consejería de Agricultura y Ganadería CyL, 2020) described in Abarquero et al. (2023).

Briefly, for each cheese batch and replicate, 220 L of sheep's milk was pasteurised, cooled to 30-32°C and calcium chloride (0.2 g L^{-1}) was then added. Cheeses were manufactured using the following combination of SLAB and NSLAB: (control batch "T") 1% KFP R-604 (commercial LAB starter culture; Chr. Hansen SL, Madrid, Spain); (experimental batch "L1") 1% KFP R-604 + 0.5% NSLAB *Lactiplantibacillus plantarum* TAUL67; (experimental batch "L2") 1% KFP R-604 + 0.5% NSLAB *Lb. plantarum* TAUL67 + 0.5% NSLAB *Lacticaseibacillus paracasei* TAUL1752; (experimental batch "L3") 1% KFP R-604 + 0.5% NSLAB *Leuconostoc*

mesenteroides TAUL1342 + 0.5% NSLAB *Lb. plantarum* TAUL67; (experimental batch "L4") 1% SLAB *Lc. lactis* GE44 + 0.5% NSLAB *Ln. mesenteroides* TAUL1342 + 0.5% NSLAB *Lb. plantarum* TAUL67 (Fig. 1). The concentration of the autochthonous cultures was 10^6 CFU mL^{-1}. After 30 min of acidification, 0.05 mL L^{-1} of chymosin (CHY-MAX Extra, 100% chymosin; 600 IMCU mL^{-1}; Chr. Hansen SL) was added while maintaining the milk temperature at around 32 °C. After 30-35 min of coagulation, the curd was cut into grains the size of maize-rice grains. The whey was then drained off, and the curd was transferred to cylindrical moulds (15 cm high, 21 cm in diameter). After pressing for 2 h, the cheeses were immersed in brine (18 °Baume, 8 °C and pH 5.4) for 16 h to salt those. At each sampling point (7, 30, 90, 180 and 240 days) and from control batch (T) and each experimental batches (L1, L2, L3 and L4), two whole cheeses of approximately 3.25 kg were sampled. A portion of the whole cheese was used for texture profile (TPA) and microstructure analysis. The rest of the cheese was crushed, packaged, and stored at -30 °C until analysis.

2.2. Nitrogen fraction analysis

pH 4.4-soluble nitrogen (pH 4.4-SN) and 12% trichloroacetic acid soluble nitrogen (TCA-SN) of the cheeses were determined at each ripening time following FIL-IDF standard 224 (2011), using a preparation method described previously by Bütikofer, Rüegg and Ardö (1993). The pH 4.4-SN and TCA-SN were expressed as percentage of total nitrogen (TN). From the values of pH 4.4-SN and TCA-SN, the percentages of polypeptide nitrogen (PP-N) and casein nitrogen (CN-N) were determined. PP-N was determined by the difference between pH 4.4-SN and TCA-SN; while the CN-N was determined by the difference between the TN and pH 4.4-SN.

2.3. Determination of free amino acids and biogenic amines

FAA and BA were measured for cheeses at every stage of ripening, using the method described by Renes et al. (2019). The method involved homogenising 1 g of cheese with 10 mL of 0.1 M HCl-0.2% 3,3′ thiodipropionic acid (Sigma-Aldrich, Madrid, Spain) using an IKA T-18 Ultra-Turrax (IKA-Werke GmbH & Co, Staufen, Germany) for 2 min at 20,000 rpm. The mixture was subjected to 30 min of ultrasonication in a Bransonic 221 ultrasonic bath (Branson Ultrasonics S.A, Danbury, USA), followed by centrifugation at 5,000 ×*g* for 20 min. The supernatant was clarified using ultrafiltration inserts (Amicon Biomax 5 K; Millipore, MA, USA) via centrifugation at 3,500 ×*g* for 1 h. Afterwards, 20 µL of this sample were treated with diethyl ethoxymethylenemalonate (DEEMM; Sigma-Aldrich, St. Louis, MO., USA) using the process described by Redruello et al. (2013). FAA and BAs were identified and quantified by ultra-high performance liquid chromatography (UHPLC) according to Redruello et al. (2013).

2.4. Microstructure analysis

The cheeses were analysed with Confocal Scanning Laser Microscopy (CSLM) at 7, 30, 90, 180, and 240 days of ripening, as described by Auty et al. (2001) to visualize the changes in the fat and protein distributions at the microstructural level. Cheese samples were cut to 10 mm × 10 mm × 1 mm size using a surgical blade and stained with 50 µL of a probe mixture of Nile Red (Sigma-Aldrich), 0.02 g L^{-1} and Fast Green FCF (Sigma-Aldrich), 0.1 g L^{-1}. It was then examined using a Zeiss LSM800

confocal laser scanning microscope (Carl Zeiss, Welwyn Garden City, Herts, UK), with dual excitation using 488 nm/633 nm for Nile Red/Fast Green FCF.

2.5. Texture profile analysis

Texture profile analyses (TPA) were conducted at room temperature (20 ± 2 °C) on eight cube-shaped cheese samples (20 mm x 20 mm x 20 mm) and at each ripening time, to measure hardness, adhesiveness, springiness, cohesiveness, chewiness and gumminess. Texture properties were determined in two successive cycles of 80% compression with a cross-head constant speed of 0.5 mm s^{-1} using a TA-XT2i texturometer (Stable Micro Systems, Godalming, UK) equipped with a plate-plate sensor system with a stainless SMS P/75 (75 mm) probe. The textural characteristics were derived from the resulting force-time curve using Exponent software v.6,1,20,0 (Stable Micro Systems).

2.6. Statistical analysis

Statistical analysis of experimental data was performed using SPSS v.25 (SPSS, Chicago, IL, USA). The nitrogen fractions, FAA, BA and texture variables were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using the Levene test. One-way analysis of variance (ANOVA) was then used to assess the effect of co-culture and ripening time as fixed effects and their interaction. Tukey's HSD post hoc test at 5% significance level was used to compare the effect of different combinations of BAL throughout ripening.

3. Results and discussion

3.1. Changes in nitrogen fractions during ripening

The percentages of pH 4.4-SN, TCA-SN, CN-N and PP-N, expressed as a percentage of nitrogen with respect to TN, of cheeses throughout the ripening process are shown in Figure 2. Significant differences (p<0.05) were observed between the cheese batches for the different ripening times in the pH 4.4 SN fraction (Fig. 2A). In general, the percentage of pH 4.4 SN was three times higher across all cheese batches from the beginning to the end of the ripening process, with an increase from approximately 9.50-11.66% at 7 days to 31.75-33.90% after 240 days. The most important increase in all batches occurred during the first 30 days, reaching the highest values of this fraction at 240 days of ripening. It should be noted that the L3 and L4 experimental batches were those that presented the highest pH 4.4-SN values at 180 days of ripening (32.27% and 33.02%, respectively), with no significant differences (p>0.05) between these batches at 180 and 240 days. The effect of the starter culture would not be as important as reported Poveda et al. (2015) or Candioti et al. (2010), who did not observe significant differences (p>0.05) in the percentages of pH 4.4-SN between sheep cheeses made with or without lactobacilli, because the agents responsible for the formation of this nitrogenous fraction are chymosin and to a lesser extent plasmin.

As in the previous case, the percentage of TCA-SN (Fig 2B) increased significantly (p<0.05) in all batches during the ripening time, reaching values between 20.28% and 22.11% at after 240 days. In this fraction, the increase in their values was much greater than for pH 4.4-SN, doubling the content between 7 and 240 days in all

cheese batches. At the beginning of ripening, TCA-SN represented approximately 36 – 40% of pH 4.4-SN, while at the end, this percentage represented over 60%. The differences observed in secondary proteolysis between the batches could be related to the inclusion of lactobacilli strains. The proteolytic activity of lactobacilli is greater than that of lactococci because they present additional peptidases with higher expression levels (Pappa et al., 2006). Mezo-Solís et al. (2020) reported TCA-SN values 40% lower than those obtained in this work. On the other hand, they were higher than those reported for Torta del Casar cheese (Delgado et al., 2010), Antep cheese (Ertekin et al., 2023), Fiore Sardo cheese (Caboni et al., 2019) or Croatian Krk cheese (Mikulec et al., 2008).

CN-N (Fig. 2C) was significantly reduced (p<0.05) during the ripening of Castellano cheese. This decrease was more pronounced during the first 90 days of ripening, from 88.0-90.50% at 7 days to 72.93-76.14% at 90 days of ripening, which represented a decrease of 16%, while between 90 and 240 days a lower degradation of casein nitrogen was observed, which was only 7% in global terms. In parallel with the hydrolysis of caseins, there was a significant increase (p<0.05) in the percentage of PP-N during the ripening of cheeses, reaching values between 10.83% and 12.89% at the end of ripening (Fig. 2D). The increment in the percentage of PP-N occurred mainly during the first 90 days of ripening, where PP-N was up 73%, while between 90 and 240 days, the mean values remained practically constant. The greatest differences between batches were observed in this fraction, possibly due to the proteolytic activity of the autochthonous NLAB strains. L2 showed the highest values at 90 days of ripening, while L3 and L4 showed the highest values at 180 days. The inclusion of *Lb plantarum* and *Ln. mesenteroides* may accelerate the course of ripening, as these LAB were once selected for their notable proteolytic activity.

The results of the evolution of proteolysis in Castellano cheese, monitored through its nitrogen fractions during ripening, agree with what has been reported by other authors for other Spanish sheep cheese varieties, similar in production technology and ripening time, such as Zamorano (Delgado et al., 2010), Idiazábal (Santamarina-García et al., 2022), Manchego (Hayaloglu et al., 2012) or Roncal (Delgado et al., 2010). Our results were also very similar to those described by other authors for other sheep cheeses with similar maturation times such as Pecorino Romano (Addis et al., 2015) or a Mexican Manchego-type cheese (Mezo-Solís et al., 2020).

3.2. Changes in free amino acids and biogenic amines during ripening

Total FAA content increased during ripening of the cheeses, from an average value of 665 mg kg^{-1} of cheese after 7 days of ripening to average values of 19,497 mg^{-1} kg of cheese after 240 days (Fig. 3). At the end of ripening these values were like that described by other authors for ripened European cheeses (Manca et al., 2023; Pachlová et al., 2018; Redruello et al., 2020); although higher than those described for Bzyaz, Kasar and Tulum cheeses (Ayag et al., 2022) or Cremoso and Patagrás cheeses (Milesi et al., 2009). In general, the increase in FAA content occurred constantly throughout the cheese ripening process, although it was more pronounced in all cheese batches after the first 30 days of ripening. This trend agrees with those reported by other authors for cheeses with similar characteristics made with NSLAB (lactobacilli) (Pachlová et al., 2018; Poveda et al., 2015; Zhang et al., 2021).

Higher FAA content was obtained in the experimental batches where adjunct cultures (NSLAB) were used (L1, L2, L3 and L4) compared to cheeses from control batch T, which was only made with the commercial starter culture. After 90 days of ripening, there was significant differences (p<0.05) in total FAA content, especially when comparing batch L4 with the rest. Although L4 and L2 had the highest FAA contents at the end of ripening, L4 maintained higher values than L2 at 90 and 180 days of ripening. The differences between cheese batches seem to be associated with the metabolic activity of the LAB included in the starter and/or adjunct culture and its increased peptidase activity. SLAB and NSLAB, play a key role in proteolysis thanks to enzymatic activities, such as aminopeptidase or dipeptidase, which contribute to the hydrolysis of peptides into FAA (Renes et al., 2021; Santamarina-García et al., 2022). In addition, the sensory characteristics of each cheese variety are influenced by the release of FAA and its transformation into volatile and non-volatile compounds, such as esters, ketones, or aldehydes (Khattab et al., 2019).

Finally, the observed increase in FAA (Table 1) agrees with what was expected, since the amino acids formed are a consequence of the proteolytic activity of the LAB included in the cultures, which occurs during secondary proteolysis, as has been verified in the previous section. At the end of the ripening, 19 amino acids were identified (Table 1). The profile of major FAAs was very similar between the beginning and the end of maturation. The most abundant amino acids in all cheeses were glutamic acid, leucine, proline, valine, γ-aminobutyric acid (GABA) and lysine. These fives amino acids together represent more than 55% of total FAA after 240 days of ripening. These results were very similar to those described by other authors in sheep cheese with similar characteristics such as Manchego cheese (Poveda et al., 2015), Roncal type cheese (Irigoyen et al., 2007), Pecorino Sardo (Madrau et al., 2006), Fiore Sardo (Manca et al., 2023) or Zamorano-type cheese (Renes et al., 2021).

Specifically, it should be noted that in all cheeses and from the first stages of ripening, significant contents of GABA and ornithine were detected. These compounds have acquired great relevance in recent years due to their beneficial physiological effects on human health (Diana et al., 2014). After 240 days of ripening, the concentration of GABA ranged between 230 and 2,000 mg/kg while that of ornithine ranged between 600 and 750 mg/kg of cheese. These contents represented around 5.7% for GABA and 3.5% for ornithine of total FAA in Castellano cheese, being like those described by Manca et al. (2023) for Fiore Sardo cheese, although lower than those reported by Renes et al. (2021). On the other hand, the glutamic acid content presented a different behavior than that reported by other authors, since its presence was only detected after 90 days of ripening, showing significant differences (p<0.05) between the different batches. The highest concentrations of this amino acid were observed in control batch T and experimental batch L4, while in the rest its presence was only described in lower concentrations in cheeses after 180 days of ripening. It seems that there was a relationship between the use of the *Lb. plantarum* TAUL67 and glutamic acid and GABA concentrations. In fact, the strain of *Lb. plantarum* TAUL67 was selected for its high decarboxylase activity on glutamic acid (Abarquero et al., 2023a), which would explain the low contents of this amino acid in the experimental batches L1, L2 and L3 in which this strain of *Lactobacillus* was incorporated. In batch L4, which also contained this strain, the high glutamic acid content seems to be due to an inhibitory effect of *Lc. lactis* GE44 on *Lb. plantarum* strain (Abarquero et al., 2023b). The transformation of glutamic acid into GABA by the *Lb. plantarum* strain would

explain why the GABA content in L1, L2 and L3 turned out to be on the order of 3 to 5 times higher than that of T and L4. Redruello et al. (2020) reported that one of the factors most influencing the GABA content in cheeses was the type of cultures used in their production, corroborating the results obtained in our study.

Finally, regarding the presence of BA, only the presence of ethylamine was detected (Fig. 4). The presence of ethylamine in Castellano cheese increased significantly (p<0.05) with ripening time, reaching the highest values after 90 days, from which time they decreased. The absence of BA in Castellano cheese differs from that described by other authors in sheep cheeses such as Manchego (Poveda et al., 2015), Zamorano type (Renes et al., 2021) or Fiore Sardo (Manca et al., 2023). The presence of BA occurs because of the decarboxylation of FAA by some LAB strains (Pachlová et al., 2018). In this study, the autochthonous LAB strains used had been previously selected under the criterion that they were not BA producers (Abarquero et al., 2023b), which would explain the absence of some hazardous BA, such as tyramine or histamine. However, the appearance of ethylamine, a volatile BA, has rarely been described in cheeses, although its presence has been described in Pecorino Abruzzese (Martuscelli et al., 2005) or Terrincho (Pintado et al., 2008). In this last study, they associated this amine with the presence of enterococci in cheese. However, no negative effects related to the consumption of ethylamine in cheese have been described. The importance of BA is due to the negative effects that some of them, such as histamine or tyramine, have on the health of some consumers (Schirone et al., 2022). Consequently, it is a priority to control the production of BA by using SLAB and NSLAB cultures that do not produce these compounds (Gardini et al., 2016).

3.3. Evolution of the cheese microstructure during ripening

Figure 5 shows the evolution of the microstructure of the cheeses during ripening, analysed by means of CSLM images. The microstructure of hard cheeses such as Cheddar or Castellano is characterised by the arrangement of its components, including casein proteins, fat globules and the aqueous phase. On the one hand, the protein phase is formed by the aggregation of casein micelles that form a three-dimensional network, while the fat phase consists of intact fat globules, aggregated fat globules, fused fat globules and non-globular fats (Lamichhane et al., 2018; Ong et al., 2022). This arrangement, along with the changes that occur during ripening, determines the final texture of the cheese, as well as flavour and other sensory attributes. Initially, the microstructure is influenced by factors such as casein concentration, properties of casein micelles, coagulation conditions and pH during the ripening process. However, during ripening, the protein network structure is altered by complex physical and biochemical changes in the cheese matrix, such as proteolysis, casein demineralisation and hydration of the casein network (Lamichhane et al., 2018).

In general, the microstructure of all the cheeses showed similar behaviour during the ripening process. During the first seven days of ripening, a dense and cohesive protein matrix with unevenly distributed fat globules was observed. In other similar cheeses, such as Cheddar or Zamorano, a comparable distribution was observed, accompanied by a slight linear orientation related to the pressing of the cheese (Aldalur et al., 2019; Renes et al., 2019). However, as proteolysis progressed, there were changes in the protein matrix, resulting in an amorphous structure during ripening. Figure 5

illustrates the changes in structure after 30 days of ripening, corresponding to changes in nitrogen fractions (Fig. 2). A significant increase in the pH 4.4-SN and TCA-SN fractions was observed at this time. On the other hand, the fat phase also underwent changes, evolving from small fat globules at the beginning of ripening to larger aggregates, possibly as a result of fusion between fat globules. In addition, ripening results in the release of free fatty acids due to the lipolytic activity of the fat globules (Rogers et al., 2010; Zisu & Shah, 2005). Finally, it should be noted that although all cheeses showed similar development the loss of initial structure did not follow the same pattern for all batches at the same ripening times, which could be an effect of the NSLAB used in each batch. The protein/fat matrix serves as a support for microorganisms and its activity during ripening influences the microstructure (Pereira et al., 2009).

3.4. Texture profile analysis during ripening

Figure 6 shows the results of the different parameters evaluated in the TPA (hardness, adhesiveness, elasticity, cohesiveness, chewiness and gumminess) for the cheeses made with different starter and NSLAB cultures.

Firstly, all the textural parameters were significantly influenced by the ripening time ($p<0.05$). Hardness values increased significantly ($p<0.05$) in all cheeses during the first six months of ripening and remained constant until the end of ripening, when they reached average values of around 258 N. A very similar behavior was observed for chewiness and gumminess values, with the highest indices corresponding to cheeses aged 180 and 240 days. These increases in hardness, chewiness and gumminess were related to the decrease in moisture content of the cheeses during ripening. Several authors have found that the hardness of cheese is primarily determined by moisture content (Tejada et al., 2007; Tomar, 2019). As the water content decreases during ripening, the protein concentration in the cheese increases, leading to a greater number of protein interactions. This contributes to the strengthening of the three-dimensional para-*kappa*-casein network, requiring more force to deform and chew the cheese (Álvarez & Fresno, 2021). On the other hand, the springiness and cohesiveness values of Castellano cheese decreased with ripening, while the adhesiveness values increased, being more pronounced in the case of cohesiveness and adhesiveness. In the early stages of ripening, the flexibility of the interactions in the para-*kappa*-casein matrix will be responsible for the elastic character, confirming the higher springiness values (around 45%) in the 7-day Castellano cheese. However, there were changes in pH, salt/moisture ratio and colloidal calcium concentration during ripening (Abarquero et al., 2023b), which would have influenced the degree of proteolysis, promoting the interaction of proteins with water. As a result, the cheese loses its elastic modulus and becomes more viscous (O'Mahony et al., 2005; O'Mahony et al., 2006). Cohesiveness values were significantly reduced ($p<0.05$) during the first month of ripening, from an initial average of 15-16% to around 12% after 30 days. From then on, their values remained constant, although a slight increase was observed after 240 days. These results were like those reported by Sallami et al. (2004) and O'Mahony et al. (2005) for Cheddar cheese. This decrease in cohesiveness seems to be related to a reduction in protein interactions because of the increase in the salt/moisture ratio and the loss of water, resulting in less elastic and cohesive and crumblier cheeses (Pastorino et al., 2003a). Regarding adhesiveness, its values increased significantly ($p<0.05$) during the first six months of ripening, with no significant changes thereafter. This behavior could be related to the increase in ionic strength and the degree of proteolysis during ripening, which

contribute to a greater interaction of the protein matrix with water and other non-protein components (Diezhandino et al., 2016; Pastorino et al., 2003b; Visser, 1991). As a result, Castellano cheese becomes more viscous and stickier, requiring more force to separate it from the texture probe.

Regarding the effect of the cultures used in the production of the Castellano cheeses, significant differences (p<0.05) were observed between them at almost all stages of ripening for the parameters of hardness, chewiness, gumminess and adhesiveness, but not for elasticity and cohesiveness (p>0.05). The differences in hardness, chewiness and gumminess were more pronounced during the first month of ripening. At the beginning of ripening, experimental batches L1 and L2 had the highest values for these parameters, which could be linked to a higher dry matter content. However, as ripening progressed, the differences between the cheeses decreased until similar values for hardness, chewiness and gumminess were reached after 240 days for experimental batches L1, L2, L3 and L4 (between 198.77 and 216.84 N). Only cheeses from batch control T (produced with the commercial starter culture without adjunct strains) differed significantly (p<0.05) from the others, with higher hardness values. In contrast, the cheese from batch L4, made with the autochthonous SLAB (*L. lactis* GE 44) and NSLAB (*Ln. mesenteroides* TAUL 1342 and *Lb. plantarum* TAUL 67), showed the lowest hardness values during the first six months of ripening, being significant (p<0.05) with the rest of the batches. Our results agree with those reported by Fernández et al. (2019) in Zamorano sheep's cheese made with a mixture of autochthonous cultures with lactobacilli and enterococci. Adhesiveness in the cheeses increased significantly (p<0.05) with ripening time up to 180 days, before decreasing slightly. Cheeses from control T showed the highest adhesiveness (-11.19 N·s), while batches L1, L2 and L4 had the lowest adhesiveness (-7.56, -6.96 and -7.74 N·s, respectively). These results of Castellano cheese could be due to the increase in fat content because of water loss during ripening, as well as the degree of primary and secondary proteolysis developed.

Differences between treatments would be expected to be driven by changes in chemical composition, pH, salt/moisture ratio or proteolysis, all of which play key roles in established protein interactions in the para-*kappa*-casein network (Pastorino et al., 2003a; O`Mahoney et al., 2005; Upreti et al., 2006). However, all cheeses showed similar values of moisture, fat, protein, pH, salt/humidity (Abarquero et al., 2023b), as well as the same degree of primary (pH 4.4-SN) and secondary (TCA-SN) proteolysis at all stages of ripening. Therefore, we consider that the differences between cheese batches in the texture parameters would be associated with the greater or lesser homogeneity of the structure of the cheeses. Both lactobacilli and/or *Leuconostoc* used in the production of experimental batches L1, L2, L3 and L4 were heterofermentative, contributing to gas production during cheese ripening. In addition, there was the presence of a significant number of mechanical holes in the cheese. Both aspects contributed to the formation of a more heterogeneous protein matrix (see Fig. 5) in the batches L1, L2, L3 and L4 compared to control T, which showed less resistance to deformation forces. Likewise, the impact of NSLAB strains on cheese proteolysis should also be considered. Cheeses from control T exhibited higher levels of hardness, chewiness and gumminess than the other batches. Greater proteolysis in the cheeses possibly led to a softer texture, as indicated by Figure 2, where significant differences (p<0.05) were noticed between cheeses from control T and the remaining experimental batches in several nitrogen fractions.

4. Conclusion

The utilization of autochthonous NSLAB during the production of PGI Castellano cheese significantly impacted several proteolysis parameters, consequently affecting the cheese's microstructure and texture. The inclusion of autochthonous lactobacilli and *Leuconostoc* accelerated proteolysis, which may have an economic impact. The FAA levels were significantly higher in the cheeses containing NSLAB strains, especially in experimental batch L4 which included wild SLAB and NSLAB. The liberation of FAA holds great importance as they are the precursors for various aroma and flavour compounds in cheese. Therefore, the use of these autochthonous LAB strains increased the sensory complexity of the cheese. The inclusion of *Lb. plantarum* TAUL67 is responsible for converting glutamic acid into GABA, which is noteworthy due to the health benefits of this compound. Furthermore, the selection of autochthonous LAB strains was based on their lack of biogenic amine production, resulting in the absence of these compounds in the cheeses, enhancing its safety for consumption. In terms of texture, the inclusion of NSLAB strains proved to be positive, as the experimental cheese batches containing them showed lower values for hardness, chewiness and gumminess compared to the cheeses from control T.

The results of this research will provide a better understanding of the evolution of the proteolysis process in sheep's milk cheeses, particularly PGI Castellano during ripening. Furthermore, this research will provide information on the possible use of autochthonous NSLAB to produce cheeses with pasteurised milk, thereby improving quality and reducing ripening time, with significant industrial and economic implications.

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CRediT authorship contribution statement

Daniel Abarquero: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing. **Cristina Duque:** Investigation, Validation. **Raquel Bodelón:** Investigation. **Inés López:** Conceptualization, Resources. **Julio Muñoz:** Conceptualization, Resources. **José María Fresno:** Writing - review & editing, Supervision. **María Eugenia Tornadijo:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights

Autochthonous cultures improve proteolytic activity and texture parameters in PGI Castellano cheese

GABA levels were significantly increased by including the *Lb. plantarum* strain.

No hazardous biogenic amines were found in any of the cheese batches.

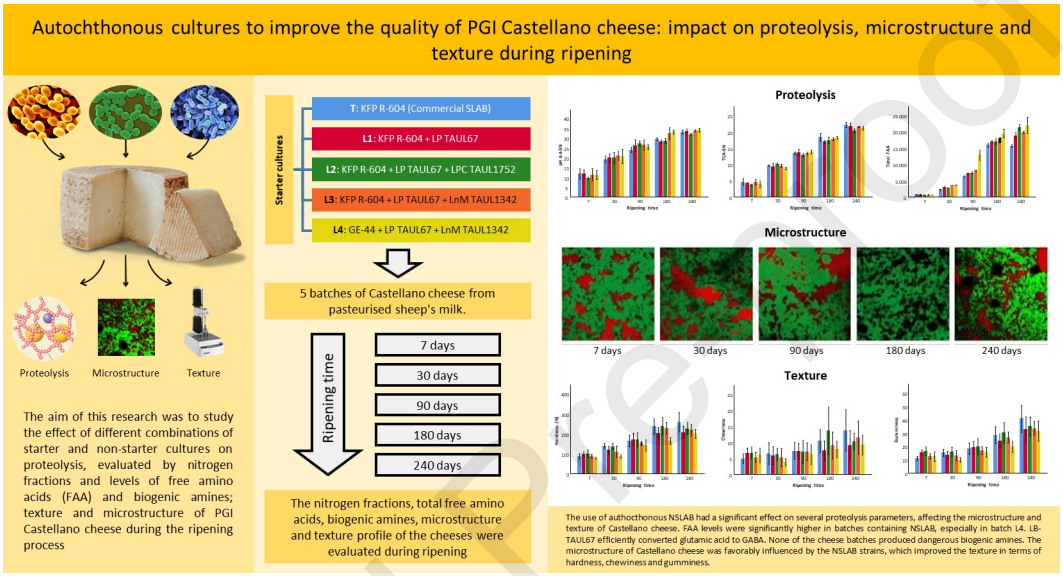


Table 1. Concentration (mg kg^{-1} of cheese) of each amino acid after 7, 30, 90, 180 and 240 days of ripening in five batches of PGI Castellano cheese made with different starter and/or non-starter cultures.

Batch	ASP	GLU	ASN	SER	GLN	HIS	GLY	THR	GABA	ALA
Ripening time = 7 days										
T	nd[1]	nd	nd	nd	nd	nd	nd	nd	33.51 ± 2.98[a]	8.91 ± 1.68[a]
L1	nd	nd	nd	nd	nd	nd	nd	nd	100.54 ± 2.98[c]	nd
L2	nd	nd	nd	nd	nd	nd	nd	nd	106.99 ± 23.20[c]	nd
L3	nd	nd	nd	nd	nd	nd	nd	nd	76.05 ± 15.40[b]	15.59 ± 3.83[b]

L4	nd	nd	nd	nd	nd	nd	nd	nd	25.78 ± 0.20[a]	6.68 ± 2.57[a]
Ripening time = 30 days										
T	nd	nd	nd	nd	295.93 ± 8.98[a]	nd	nd	25.31 ± 4.62[a]	68.32 ± 21.21[a]	49.00 ± 9.03[a]
L1	nd	nd	257.63 ± 21.43[a]	nd	365.35 ± 48.23[b]	nd	nd	43.18 ± 3.33[b]	290.03 ± 37.17[c]	72.39 ± 7.63[b]
L2	nd	nd	nd	nd	297.76 ± 17.30[a]	nd	nd	nd	349.32 ± 35.08[c]	67.93 ± 7.00[b]
L3	nd	nd	280.75 ± 22.29[a]	60.43 ± 9.10	299.59 ± 10.06[a]	nd	41.29 ± 11.58[a]	53.60 ± 9.92[b]	286.16 ± 44.05[c]	83.52 ± 9.85[b]
L4	nd	nd	355.07 ± 13.79[b]	nd	359.87 ± 10.60[b]	100.85 ± 19.19	58.18 ± 12.29[b]	55.09 ± 8.86[b]	146.95 ± 29.92[b]	71.27 ± 10.20[b]
Ripening time = 90 days										
T	nd	399.09 ± 51.45[a]	459.11 ± 30.75[a]	42.04 ± 9.20[a]	507.84 ± 27.01[a]	65.94 ± 4.48[a]	41.29 ± 3.06[a]	77.43 ± 4.86[a]	123.74 ± 15.18[a]	131.41 ± 5.75[a]
L1	nd	nd	540.03 ± 21.21[ab]	nd	632.06 ± 36.20[a]	81.46 ± 14.85[a]	65.69 ± 2.18[b]	114.65 ± 22.48[a]	827.54 ± 41.93[d]	191.54 ± 8.36[b]
L2	nd	12.87 ± 2.75[a]	589.58 ± 35.55[b]	10.51 ± 1.08[a]	635.71 ± 20.82[a]	85.34 ± 11.74[a]	63.81 ± 10.58[ab]	95.30 ± 19.33[a]	768.24 ± 57.72[cd]	181.52 ± 11.31[b]
L3	nd	51.50 ± 6.13[a]	685.36 ± 39.77[c]	nd	661.28 ± 18.56[a]	108.61 ± 9.01[a]	88.21 ± 5.25[b]	111.67 ± 13.21[a]	690.90 ± 56.50[c]	189.32 ± 21.05[b]
L4	56.57 ± 8.74	1454.75 ± 75.60[b]	1010.70 ± 75.08[d]	152.38 ± 47.10[b]	933.47 ± 168.69[b]	240.49 ± 56.02[b]	167.03 ± 29.72[c]	196.55 ± 25.02[b]	293.89 ± 34.38[b]	278.41 ± 20.58[c]
Ripening time = 180 days										
T	94.54 ± 8.48[a]	2177.52 ± 296.46[cd]	1119.70 ± 44.88[a]	249.59 ± 40.59[b]	1026.63 ± 31.13[a]	174.55 ± 18.47[a]	145.45 ± 17.17[a]	217.39 ± 2307[a]	260.38 ± 44.65[a]	367.50 ± 30.32[a]
L1	297.83 ± 4.39[c]	91.96 ± 61.40[a]	1306.32 ± 29.05[b]	nd	1253.15 ± 103.77[b]	193.94 ± 16.76[a]	175.48 ± 3.14[a]	279.93 ± 32.26[ab]	1910.30 ± 54.16[d]	485.54 ± 25.98[b]

L2	194.67 ± 11.65^{b}	976.58 ± 253.80^{ab}	1352.56 ± 37.72^{bc}	86.70 ± 11.72^{a}	1296.99 ± 86.88^{b}	188.12 ± 13.05^{a}	167.97 ± 10.09^{a}	263.55 ± 15.48^{ab}	1438.52 ± 51.42^{c}	476.63 ± 37.39^{b}
L3	173.04 ± 20.47^{b}	1451.07 ± 178.93^{bc}	1377.33 ± 68.14^{bc}	74.88 ± 13.82^{a}	1220.27 ± 52.98^{b}	219.16 ± 13.25^{a}	182.98 ± 4.14^{a}	251.64 ± 39.02^{ab}	1041.51 ± 53.22^{b}	443.22 ± 33.66^{b}
L4	189.68 ± 25.06^{b}	2742.14 ± 679.14^{d}	1423.57 ± 29.45^{c}	302.13 ± 37.01^{c}	1218.44 ± 81.23^{b}	349.10 ± 47.92^{b}	268.38 ± 41.16^{b}	300.78 ± 42.37^{b}	323.54 ± 39.86^{a}	472.18 ± 43.92^{b}
Ripening time = 240 days										
T	86.52 ± 22.41^{a}	2309.94 ± 79.69^{bc}	1160.99 ± 46.83^{a}	253.53 ± 15.09^{b}	984.62 ± 27.58^{a}	199.76 ± 3.88^{a}	151.08 ± 4.72^{a}	209.95 ± 11.27^{a}	233.31 ± 26.30^{a}	404.25 ± 19.03^{a}
L1	382.69 ± 18.98^{c}	404.61 ± 41.92^{a}	1474.77 ± 62.74^{b}	42.04 ± 9.49^{a}	1286.03 ± 89.09^{b}	248.25 ± 32.91^{ab}	207.38 ± 16.46^{b}	308.22 ± 32.39^{b}	2005.68 ± 161.30^{d}	562.38 ± 27.34^{b}
L2	381.03 ± 51.85^{c}	1423.48 ± 124.73^{ab}	1654.78 ± 30.59^{c}	235.14 ± 59.13^{b}	1479.67 ± 80.33^{c}	306.43 ± 30.86^{c}	262.75 ± 32.19^{c}	348.42 ± 29.36^{b}	1776.24 ± 128.82^{c}	644.79 ± 43.53^{c}
L3	279.53 ± 18.82^{b}	2131.55 ± 67.52^{bc}	1497.89 ± 28.22^{b}	187.85 ± 28.26^{b}	1220.27 ± 27.34^{b}	287.04 ± 28.33^{bc}	243.98 ± 27.58^{bc}	296.31 ± 8.93^{b}	899.72 ± 40.92^{b}	525.63 ± 21.21^{b}
L4	301.16 ± 38.86^{b}	3463.07 ± 649.97^{c}	1577.16 ± 109.60^{bc}	352.05 ± 82.67^{c}	1247.67 ± 25.30^{b}	393.70 ± 27.15^{d}	284.33 ± 32.23^{c}	339.49 ± 49.15^{b}	281.00 ± 19.06^{a}	573.52 ± 53.17^{b}

Table 1. Continued.

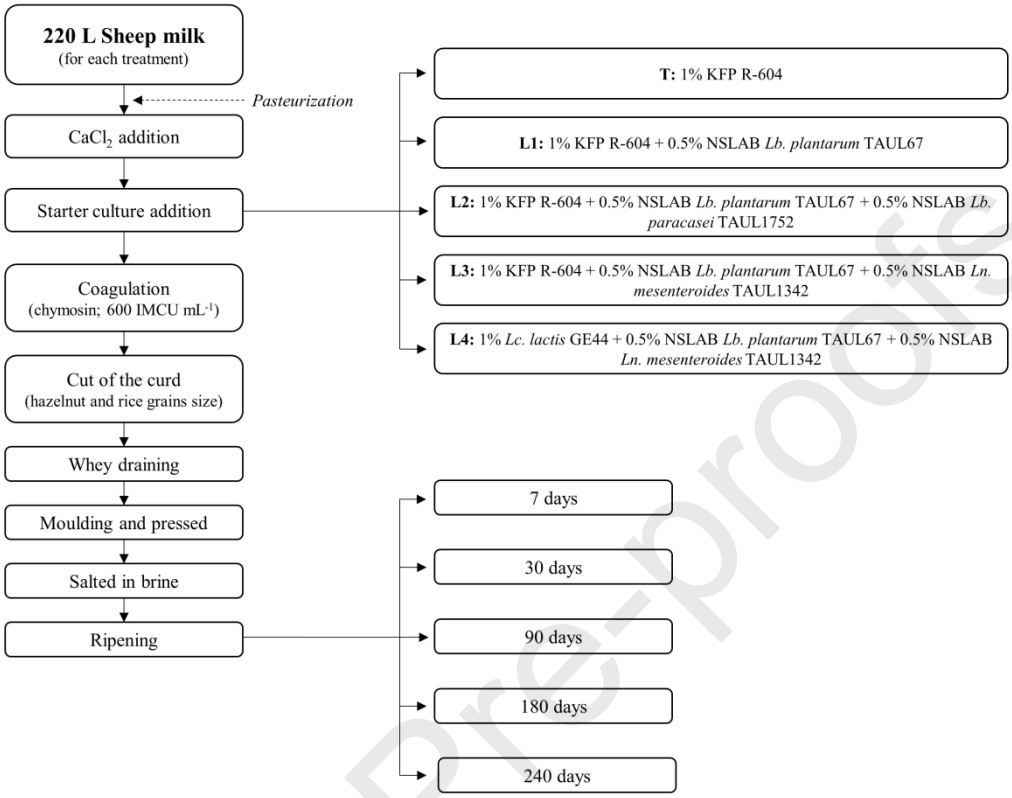
Batch	PRO	TYR	VAL	MET	TRP	ILE	LEU	PHE	ORN	LYS
Ripening time = 7 days										

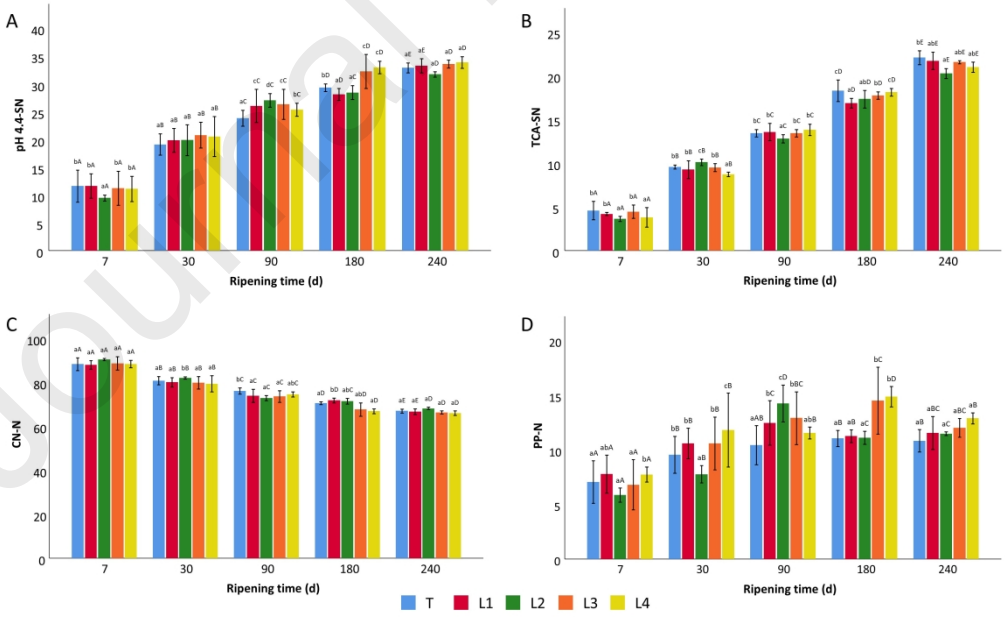
T	306.53 ± 45.66	nd	98.11 ± 11.74^{b}	nd	nd	nd	209.87 ± 16.84^{b}	nd	42.95 ± 19.08	nd
L1	323.80 ± 15.14	nd	73.22 ± 7.05^{a}	nd	nd	nd	162.32 ± 12.42^{a}	nd	11.56 ± 3.30	nd
L2	312.29 ± 32.68	nd	67.36 ± 4.42^{a}	nd	nd	nd	155.76 ± 7.61^{a}	nd	9.91 ± 3.82	nd
L3	287.83 ± 23.50	nd	95.19 ± 4.88^{b}	nd	nd	nd	198.39 ± 6.99^{b}	nd	24.78 ± 6.27	nd
L4	270.56 ± 15.44	nd	87.86 ± 2.06^{b}	nd	nd	nd	195.12 ± 8.09^{b}	nd	23.13 ± 3.82	nd
Ripening time = 30 days										
T	390.00 ± 16.27^{a}	45.30 ± 4.20^{b}	330.95 ± 17.30^{a}	109.42 ± 9.30^{a}	nd	6.56 ± 1.60^{a}	500.09 ± 23.81^{a}	194.10 ± 10.38^{a}	152.83 ± 8.77^{a}	95.02 ± 21.74^{a}
L1	492.18 ± 37.43^{b}	nd	374.88 ± 19.51^{b}	128.69 ± 7.09^{b}	nd	nd	544.36 ± 40.89^{a}	175.51 ± 32.47^{a}	151.98 ± 8.95^{a}	133.40 ± 18.10^{ab}
L2	510.89 ± 26.11^{b}	22.65 ± 5.23^{a}	377.81 ± 12.30^{b}	128.69 ± 9.57^{b}	nd	nd	539.44 ± 22.20^{a}	169.32 ± 29.60^{a}	150.33 ± 18.86^{a}	126.09 ± 24.15^{ab}
L3	506.57 ± 45.16^{b}	27.18 ± 1.54^{a}	459.82 ± 12.37^{c}	123.10 ± 3.24^{b}	nd	42.63 ± 3.41^{b}	701.76 ± 20.19^{b}	282.89 ± 21.05^{b}	191.63 ± 8.95^{b}	148.02 ± 25.74^{b}
L4	469.15 ± 66.22^{b}	24.91 ± 4.29^{a}	452.50 ± 29.86^{c}	129.33 ± 4.61^{b}	nd	50.28 ± 5.35^{c}	690.28 ± 45.18^{b}	299.63 ± 33.88^{b}	165.20 ± 14.06^{a}	144.36 ± 17.09^{b}
Ripening time = 90 days										
T	654.80 ± 15.14^{a}	158.54 ± 7.86^{a}	770.27 ± 21.89^{a}	199.57 ± 7.14^{a}	nd	111.49 ± 10.71^{a}	1188.73 ± 71.12^{a}	633.92 ± 40.67^{b}	373.35 ± 27.77^{a}	307.00 ± 17.90^{a}
L1	854.84 ± 31.63^{b}	149.48 ± 10.04^{a}	818.59 ± 28.13^{a}	277.90 ± 23.89^{b}	nd	150.85 ± 10.75^{ab}	1192.01 ± 84.05^{a}	534.80 ± 35.91^{a}	333.70 ± 41.27^{a}	471.46 ± 25.13^{b}
L2	831.81 ± 65.19^{b}	178.93 ± 24.44^{ab}	842.02 ± 69.35^{a}	257.39 ± 11.20^{b}	nd	154.12 ± 16.65^{ab}	1305.14 ± 47.10^{a}	582.29 ± 53.60^{ab}	328.75 ± 45.22^{a}	453.19 ± 35.41^{b}
L3	876.43 ± 34.34^{b}	212.90 ± 10.51^{b}	953.32 ± 26.94^{b}	266.71 ± 28.17^{b}	nd	193.48 ± 12.56^{b}	1475.66 ± 33.69^{b}	714.45 ± 31.74^{c}	381.61 ± 39.05^{a}	464.15 ± 34.02^{b}

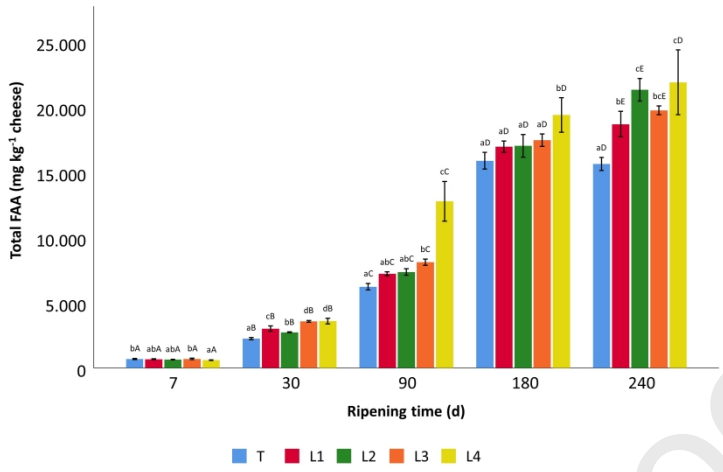
L4	1152.74 ± 81.61^{c}	371.44 ± 34.94^{c}	1331.13 ± 87.47^{c}	348.78 ± 29.79^{c}	114.88 ± 15.46	414.83 ± 74.62^{c}	1977.39 ± 117.19^{c}	1032.44 ± 35.34^{d}	501.27 ± 46.89^{b}	772.98 ± 42.02^{c}
Ripening time = 180 days										
T	1431.93 ± 50.01^{a}	539.04 ± 7.90^{a}	1514.18 ± 19.76^{a}	442.03 ± 21.43^{a}	180.40 ± 17.36^{b}	470.57 ± 24.11^{a}	2492.23 ± 37.21^{a}	1298.81 ± 71.21^{ab}	763.22 ± 37.96^{c}	952.06 ± 36.27^{a}
L1	1862.76 ± 44.50^{b}	520.92 ± 23.39^{a}	1585.93 ± 58.54^{ab}	611.76 ± 24.34^{c}	168.49 ± 5.90^{b}	601.74 ± 36.77^{b}	2500.43 ± 47.50^{a}	1195.56 ± 87.25^{a}	612.89 ± 40.08^{ab}	1335.81 ± 33.97^{bc}
L2	1544.18 ± 34.51^{a}	591.13 ± 13.09^{b}	1584.47 ± 49.31^{ab}	585.65 ± 26.60^{bc}	160.83 ± 5.11^{b}	606.66 ± 57.85^{b}	2525.02 ± 37.58^{a}	1195.56 ± 76.66^{a}	540.20 ± 42.04^{a}	1284.64 ± 72.11^{bc}
L3	1801.78 ± 56.27^{b}	591.13 ± 31.07^{b}	1666.47 ± 47.67^{bc}	542.75 ± 29.23^{b}	nd	609.94 ± 38.08^{b}	2654.55 ± 87.45^{b}	1325.65 ± 57.96^{ab}	636.02 ± 43.96^{b}	1233.48 ± 65.69^{b}
L4	1924.11 ± 106.12^{b}	656.81 ± 36.87^{c}	1717.73 ± 20.12^{c}	555.81 ± 39.86^{bc}	125.09 ± 15.47^{a}	719.80 ± 55.25^{c}	2670.95 ± 63.23^{b}	1358.69 ± 14.91^{b}	713.66 ± 45.62^{c}	1394.29 ± 91.94^{c}
Ripening time = 240 days										
T	1309.60 ± 47.07^{a}	552.63 ± 24.53^{a}	1443.89 ± 15.02^{a}	492.39 ± 10.55^{a}	nd	519.76 ± 9.84^{a}	2415.17 ± 86.05^{a}	1189.37 ± 78.65^{a}	703.75 ± 11.73^{b}	1058.05 ± 62.98^{a}
L1	2003.26 ± 37.10^{b}	597.93 ± 44.60^{a}	1656.22 ± 71.90^{b}	693.83 ± 65.89^{bc}	nd	726.35 ± 12.50^{b}	2644.31 ± 36.77^{ab}	1255.44 ± 44.06^{b}	594.72 ± 49.15^{a}	1628.19 ± 119.57^{bc}
L2	1994.63 ± 76.19^{b}	731.55 ± 45.27^{bc}	1821.70 ± 49.92^{c}	744.18 ± 31.04^{c}	nd	787.02 ± 15.65^{b}	2957.88 ± 190.76^{b}	1404.12 ± 59.52^{b}	622.80 ± 34.60^{a}	1787.17 ± 25.49^{c}
L3	1847.84 ± 59.04^{b}	672.67 ± 20.09^{b}	1779.23 ± 26.36^{c}	613.63 ± 7.14^{b}	nd	729.63 ± 6.28^{b}	2880.82 ± 75.42^{b}	1435.09 ± 46.42^{b}	763.22 ± 44.00^{b}	1492.97 ± 16.21^{b}
L4	2233.52 ± 193.18^{c}	761.00 ± 33.17^{c}	1865.63 ± 90.38^{c}	665.85 ± 61.95^{bc}	nd	887.04 ± 56.34^{c}	2854.59 ± 268.04^{b}	1402.05 ± 33.16^{b}	720.27 ± 60.80^{b}	1732.35 ± 138.26^{c}

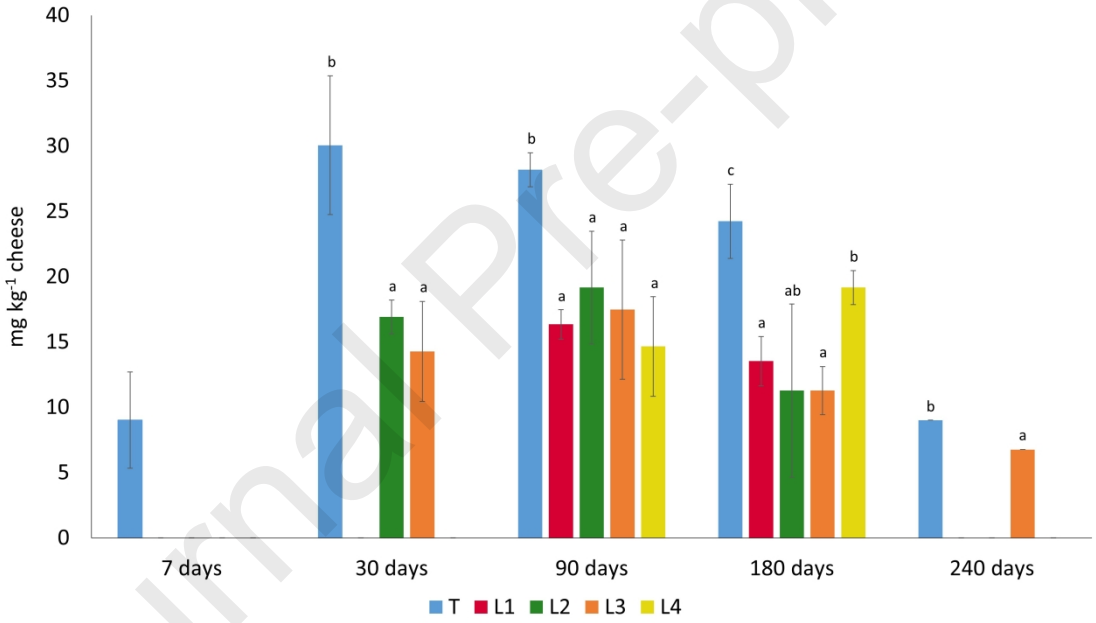
Data represent mean ± standard deviation. Different letters ($^{a–d}$) for each free amino acid indicate significant statistical differences (p<0.05) between cheese batches for the same time. ASP: aspartic acid; GLU: glutamic acid; ASN: asparagine; SER: serine; GLN: glutamine; HIS: histidine; GLY: glycine; THR: threonine; GABA: γ-aminobutyric acid; ALA: alanine; PRO: proline; TYR: tyrosine; VAL: valine; MET: methionine; ILE: isoleucine; LEU: leucine; PHE: phenylalanine; ORN: ornithine; LYS: lysine.

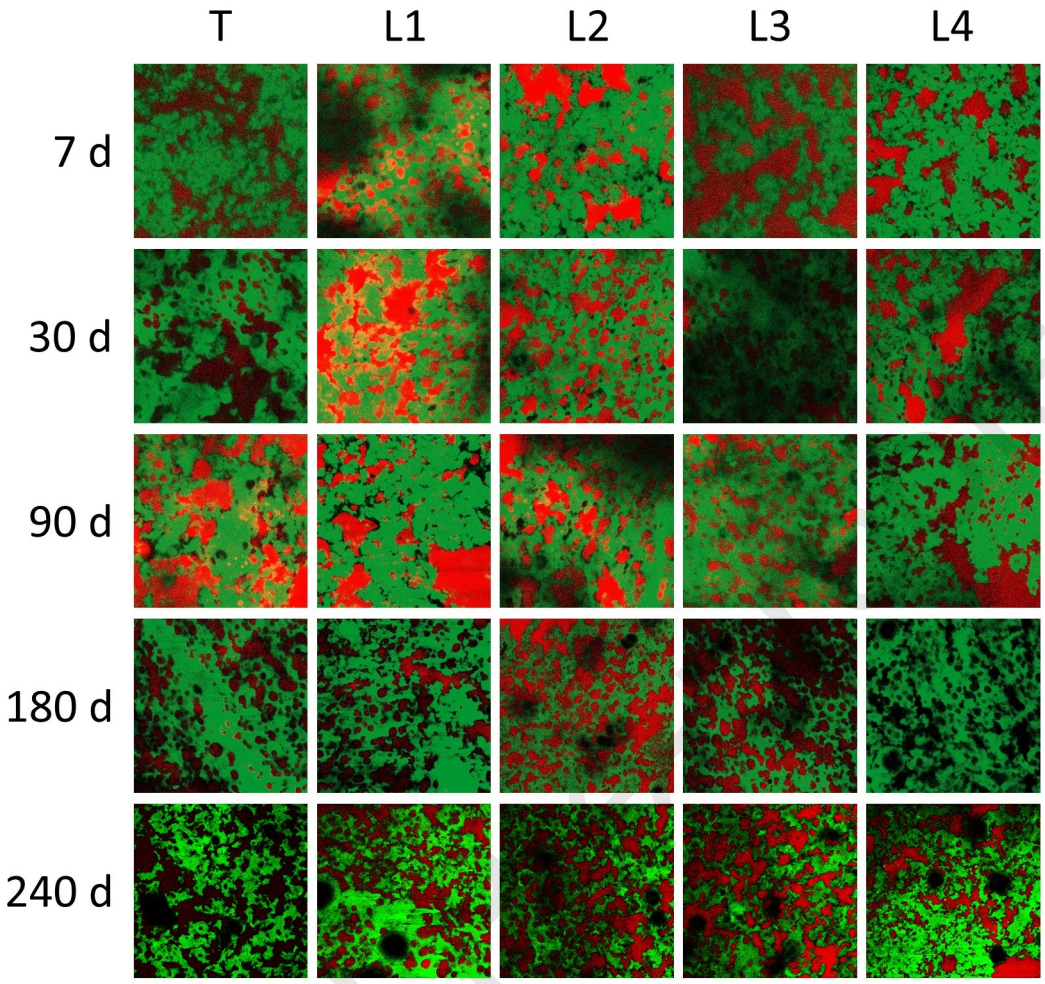
[1] nd: non detected.

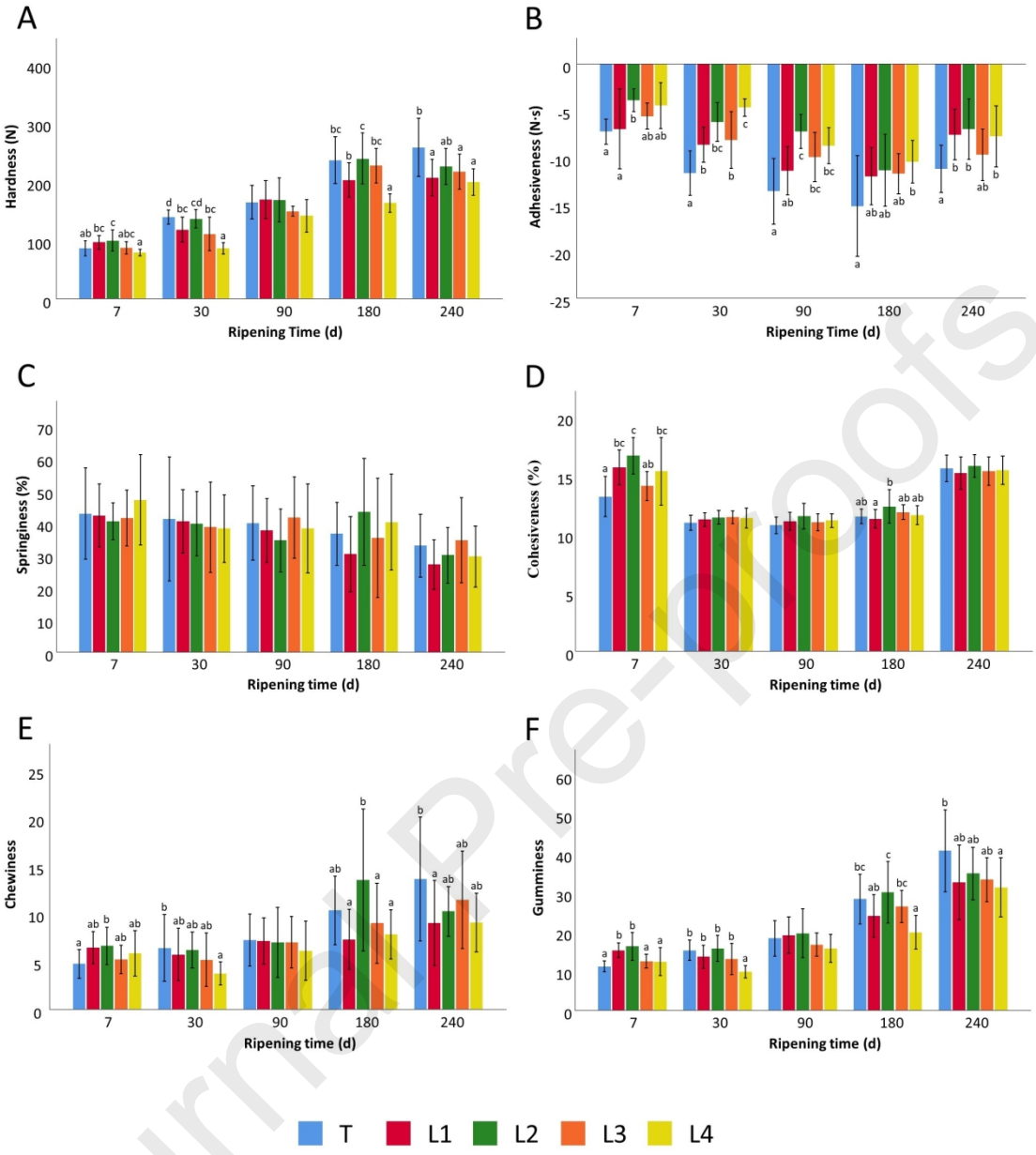












Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.