International Journal of
Dairy Technology

SDT Society of Dairy Technology

doi: 10.1111/1471-0307.13007

RESEARCH ARTICLE

Effect of autochthonous starter and non-starter cultures on the physicochemical, microbiological and sensorial characteristics of Castellano cheese

DANIEL ABARQUERO,[1] RAQUEL BODELÓN,[1] CATALINA MANSO,[2] PABLO RIVERO,[2] JOSÉ MARÍA FRESNO[1] and MARÍA EUGENIA TORNADIJO*[1]

[1]*Department of Food Hygiene and Technology, Faculty of Veterinary Science, University of León, León 24071 Spain, and* [2]*Department of Innovation and Product Development, Queserías Entrepinares, Avenida de Santander 140, Valladolid 47011 Spain*

The effect of different combinations of starter and non-starter lactic acid bacteria on the physicochemical, colour, microbiological and sensory characteristics of Castellano cheese was investigated. Five batches of Castellano cheese were produced from pasteurised sheep's milk. Significant differences in pH, titratable acidity, microbial counts and sensory attributes between the cheese batches made with different starter cultures were found. In conclusion, the incorporation of autochthonous non-starter lactic acid bacteria strains gave cheeses made with pasteurised milk better flavour and aroma characteristics than the batch made only with commercial starter culture.

Keywords Adjunct culture, Cheese ripening, Chemical composition, Lactic acid bacteria, *Lactobacillus*, Sensory analysis.

INTRODUCTION

Sheep milk production in Mediterranean and Middle East countries represents an important part of the economies (Balthazar *et al*. 2017). Practically, all the ewe's milk that is produced is used for the production of cheese mainly due to their higher protein and fat concentrations compared to milk from other species such as goat or cow (Ochoa-Flores *et al*. 2021). Many of the sheep's cheese varieties correspond to cheeses of differentiated quality certified through the Protected Geographical Indication (PGI) or Protected Designation of Origin (PDO) certification systems. These quality designations protect original and distinctive regional products, which require knowledge of all the characteristics that make the product original, specific and distinctive. (Diezhandino *et al*. 2015). The PGI Castellano cheese is an uncooked, fatty or extra-fat, enzymatically coagulated hard-cheese, made with raw or pasteurised ewe's milk from farms located in Castilla y León, Spain (European Commission 2020). Castellano cheese (Figure 1) is made by curdling the milk with animal rennet at 27–37°C for 15–55 min cutting the curds to the size of grains between hazelnut and rice. The curds are then stirred and heated to a temperature below 39°C. After straining-off the whey, the curds are transferred to cylindrical moulds, pressed and the cheeses are salted in brine. According to the PGI specifications, the minimum ripening time is 30 days for cheeses weighing 1.5 kg or less and 60 days for larger cheeses. However, it is usual for these cheeses to be marketed after at least 6 months' ripening.

As stated above, the PGI Castellano cheese is made with raw or pasteurised ewe's milk. Traditional raw milk cheeses are characterised by having organoleptic properties that are highly appreciated by consumers (Pappa *et al*. 2019). The sensory properties of cheeses made from raw milk derive primarily from the effect of the milk microbiota (Herreros *et al*. 2007). However, the production of raw milk cheese shows a higher technological and sensory variability compared to cheeses made from pasteurised milk (Pappa *et al*. 2019). Pasteurisation is the most widespread treatment for the elimination of pathogens and spoilage microorganisms present

*Author for correspondence. E-mail: metorr@unileon.es

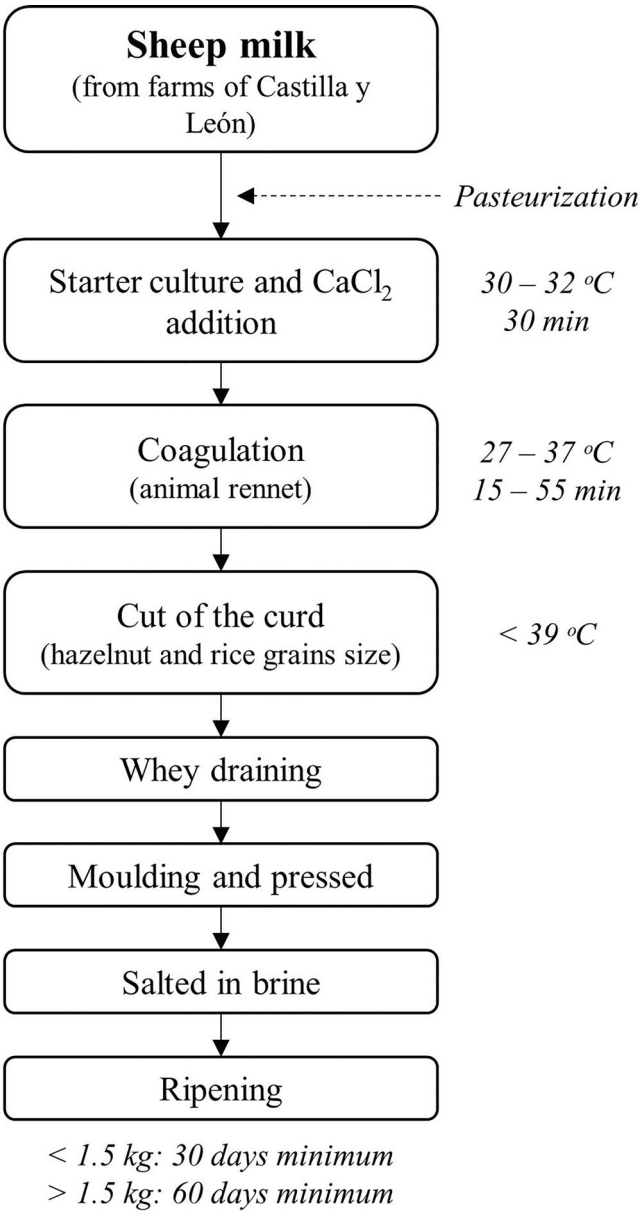

Figure 1 Flow chart of the PGI Castellano cheese production (European Commission 2020).

in milk. However, this treatment also reduces the level and diversity of the milk microbiota, which has an impact on sensory characteristics (Ruvalcaba-Gómez *et al*. 2022). Therefore, the use of starter cultures in cheese production is necessary to ensure sensory characteristics and the production of a standardised product (Özer and Kesenkaş 2019).

The use of starter cultures in cheesemaking allows the desired acidity to be controlled over a period of time and the cutting time of the curd to be limited. It also contributes to the development of the flavour and texture of the cheese (Ruvalcaba-Gómez *et al*. 2022). However, the use of commercial starter cultures reduces microbial biodiversity, which affects the final quality of the cheese (Chessa *et al*. 2021). In general, commercial starter cultures are composed of a few starter lactic acid bacteria (SLAB) species responsible for lactic fermentation. However, the microbiota of cheese, especially in raw milk cheeses, is composed of so-called non-initiating LAB (NSLAB), mainly enterococci and lactobacilli and, to a lesser extent, *Leuconostoc* spp. and thermophilic LAB (De Pasquale *et al*. 2019). NSLABs generally grow during cheese ripening and play a key role in the formation of flavouring compounds derived from various metabolic pathways including proteolysis, the main biochemical process in hard and semi-hard cheeses (Gaglio *et al*. 2020). Taking this into account, the present study was carried out to test the effect of different selected SLAB/NSLAB mixed cultures on the physicochemical, colour, microbiological and sensory characteristics of PGI Castellano cheese produced from pasteurised sheep's milk.

MATERIALS AND METHODS

LAB strains and culture design

The following LAB strains, selected for their technological and safety characteristics, were included to produce cheese from pasteurised sheep's milk: *Lactococcus lactis* GE44, *Lactiplantibacillus plantarum* TAUL67, *Lacticaseibacillus paracasei* TAUL1752 and *Leuconostoc mesenteroides* TAUL1342 (Abarquero *et al*. 2023). In addition, a commercial starter culture (KFP R-604; Chr. Hansen SL, Madrid, Spain) containing two acidifying strains (*Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) was used.

Cheese manufacture and sampling

Five batches of Castellano cheese were manufactured in duplicate at pilot scale (Queserías Entrepinares, Valladolid, Spain). For each batch and replicate, 220 L of sheep's milk was pasteurised at 65°C for 30 min and then cooled at 30–32°C. Thereafter, calcium chloride (0.2 g/L) and the starter culture were added as indicated below: (T) 1% commercial LAB starter culture; (L1) 1% commercial LAB starter culture + 0.5% NSLAB *Lb. plantarum* TAUL67; (L2) 1% commercial LAB starter culture + 0.5% NSLAB *Lb. plantarum* TAUL67 + 0.5% NSLAB *Lb. paracasei* TAUL1752; (L3) 1% commercial LAB starter culture + 0.5% NSLAB *Ln. mesenteroides* TAUL1342 + 0.5% NSLAB *Lb. plantarum* TAUL67; (L4) 1% SLAB *Lc. lactis* GE44 + 0.5% NSLAB *Ln. mesenteroides* TAUL1342 + 0.5% NSLAB *Lb. plantarum* TAUL67. Chymosin (CHY-MAX Extra, 100% chymosin; 600 IMCU/mL; Chr. Hansen SL) was added (0.05 mL/L of milk) after 30 min and then, 30–35 min were allowed for coagulation. After coagulation, the curd was cut into grains to maize–rice grain size. The whey was then drained off and the curd was transferred to cylindrical moulds (15 cm high, 21 cm in diameter). After 2 h of pressing, the cheeses were salted by immersion for 16 h in brine (18°Baume, 8°C and pH 5.4). Finally, the cheeses

were placed in a ripening chamber with a relative humidity of 80–85% and a temperature of 10°C.

Samples were taken from each batch after 7, 30, 90, 180 and 240 days of ripening. The samples (each sample corresponded to two whole cheeses of 3.25 kg) were ground, packed and stored in a freezer (−30°C) until further analysis. Physicochemical, colour and microbiological analysis parameters were carried out on fresh samples.

Chemical and physicochemical analyses

Total solids, protein and fat contents were determined according to standards 004 (FIL-IDF 2004), 20-1 (FIL-IDF 2001) and 221 (FIL-IDF 2008) respectively. Cheese pH and titratable acidity were determined according to standard 14.022 and 16.247 (AOAC 1980a, 1980b, respectively). Water activity (Aw) was instrumentally analysed using an Aqua Lab Dew Point Analyser CX-2 (Decagon Devices, Pullman, WA, USA) and NaCL content was determined according to standard 935.43 (AOAC 1990). All samples were carried out in triplicate.

Colour analyses

The colour analysis of the cheeses was carried out using a reflectance spectrophotometer (CR-5; Konica Minolta, Osaka, Japan) equipped with an 8-mm-diameter measuring glass head, a D65 illuminant and a 10° observer. For each cheese, five measurements were taken at different locations on the longitudinal surface of the cheese sample (1 cm thick). The colour measurements L^*, a^* and b^* were determined according to the CIELab colour space, where L^* corresponds to the light–dark chromaticity (0% = dark to 100% = light), a^* to the green–red chromaticity (−60% = green to 60% = red) and b^* to the blue–yellow chromaticity (−60% = blue to 60% = yellow).

Microbial counts

Fifty grams of cheese was homogenised with 200 mL of a sterile 2% (w/v) sodium citrate solution (Merck, Darmstadt, Germany) at 30–35°C for 2 min in a Masticator Classic 400 mL (IUL instruments, Barcelona, Spain). From this first dilution (1:5), decimal dilutions were prepared by mixing 10 mL of the above dilution with 90 mL of sterile 0.1% (w/v) peptone water (Oxoid, Unipath Ltd., Basingstoke, UK) according to FIL-IDF standard 122 B (FIL-IDF 1992).

Aerobic mesophilic bacteria were enumerated on standard Plate Count Agar (PCA; Condalab, Madrid, Spain) after incubation at 30°C for 48 h. Presumptive lactococci were enumerated on M17 agar (Difco, Detroit, MI, USA), after incubation at 30°C for 18–24 h, presumptive *Leuconostoc* were determined on Mayeux, Sandine & Elliker (MSE) agar (Biokar, Beauvais, France) after incubation at 25°C for 4 days; and lactobacilli on Rogosa agar (Condalab) incubated at 30°C for 5 days. Enterococci were determined on Kanamycin Esculin Azide Agar (KAA; Condalab) after incubation at 37°C for 24 h. *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBGA; Condalab), after incubation at 37°C for 18–24 h. Finally, moulds and yeasts on oxytetracycline–glucose–yeast extract (OGYE) agar (Condalab) were incubated for 5 days at 22°C. On standard PCA, Rogosa agar, KAA and VRBGA, 1-mL volumes of each dilution were inoculated in duplicate, and mixed before solidification. Plates of Rogosa agar and VRBGA were covered with a layer of the same medium before incubation. On M17, MSE and OGYE agar, 0.1 mL volumes of each dilution was surface plated in duplicate.

Sensory evaluation of the cheeses

The sensory evaluation of cheeses made at 30, 90, 120 and 240 days of ripening was carried out through a descriptive sensory analysis. For analysis, pieces of cheese of the same dimensions (4 cm × 1.5 cm × 0.5 cm) were presented to the panel at room temperature (20 ± 2°C) and identified with a 3-digit random code. Descriptive sensory analysis of the cheeses was performed by panels of six to ten tasters trained, following the ISO 8586:2012 standard (ISO 2012). The panel assessed 24 sensory characteristics, divided into four main groups: appearance, which included colour intensity [ranging from white (1) to off-white (4) to yellowish-brown (7)], cracks presence, number of holes, holes size, hole homogeneity and crystals presence; odour, which included odour intensity, odour persistence, rancid, spicy, soap and mouldy; taste, which included taste intensity, taste persistence, sweetness, acidity, saltiness and bitterness and texture, which included elasticity, firmness, friability, adhesiveness, solubility and creaminess. These attributes were recorded on a 7-point intensity scale containing the following descriptors: 1 = non-existent, 2 = very weak, 3 = weak, 4 = moderate, 5 = strong, 6 = very strong and 7 = extremely strong.

Statistical analysis

Statistical analysis of the experimental data was performed using SPSS v.25 (SPSS Inc., Chicago, IL, USA). Physicochemical, colour and microbiological variables were analysed using a general linear model of ANOVA to investigate the effect of starter culture, ripening time and the interaction between them. Tukey's honestly significant difference *post hoc* test was applied at 5% significance level to compare cheese batches manufactured with the different cultures throughout the different ripening times. Sensory variables were analysed using the Kruskal–Wallis test by ranks at 5% significance level. The Mann–Whitney test was applied at 5% significance level to compare sheep cheeses at different times of ripening. The analysis of all sensory variables for each ripening time was performed by factor analysis with principal component extraction (FAEPC).

To estimate the relationship between the different physicochemical, colour, microbiological and sensory variables

among themselves and with the ripening time and starter culture effect variables, Spearman's rank correlation coefficient (ρ) was applied.

RESULTS AND DISCUSSION

Changes in chemical and physicochemical parameters during ripening

Figure 2 shows the evolution of the physicochemical parameters during the ripening of the five batches of Castellano cheese. In general, the evolution of the physicochemical parameters analysed during the ripening time followed a similar pattern, although statistically significant differences ($P \leq 0.05$) were found at some sampling points due to the effect of the starter culture.

Total solids (TS) increased progressively during ripening ($P \leq 0.001$) due to moisture loss, reaching an average content of 71.5 ± 0.6 g TS 100/g cheese at the end of ripening (Table 1). Nevertheless, there were no statistically significant differences ($P > 0.05$) between the cheeses made with the different starter cultures. The protein content (Figure 2a) of the cheeses showed significant differences ($P \leq 0.001$) throughout ripening, but no statistically significant differences ($P > 0.05$) were found between the cheeses made with the different starter cultures. After 240 days of ripening, the average protein content of the cheeses was 35.2 ± 0.3 g protein 100/g TS. On the other hand, the fat content (Figure 2b) of the cheeses showed significant differences ($P \leq 0.001$) throughout ripening between the cheese batches. After 240 days of ripening, batch L4 had the highest fat content (54.4 ± 1.50), while batch L2 had the lowest (52.7 ± 1.5). The evolution of the fat and protein content of the cheeses included in this study followed a similar trend to that observed in other studies (Mezo-Solís *et al*. 2020; Aldalur *et al*. 2021), although the contents in the present study were slightly higher.

The pH value (Figure 2c) of the cheeses showed significant differences ($P \leq 0.001$) throughout ripening, as well as, statistically significant differences ($P \leq 0.05$) between the cheeses made with the different starter cultures. A decrease in pH was observed from values between 5.42 and 5.53 at the beginning of ripening to values between 5.23 and 5.36 after 90 days of ripening. After 90 days of ripening, pH values increased until 180 days (pH between 5.46 and 5.56). At the end of ripening, the pH values were between 5.21 and 5.37. In general, batches L1 and L2 showed the lowest pH values (5.22 and 5.21 at the end of ripening respectively), while batches *T* and L4 showed higher values (5.37 and 5.34 at the end of ripening respectively). Cheese batches made with *Lactobacillus* strains (L1, L2, L3 and L4) showed a more significant decrease in pH than cheese made without the addition of *Lactobacillus* (T). This effect was also observed by other authors (Renes *et al*. 2019; Bancalari *et al*. 2020). The increase in pH recorded in all cheese batches after 180 days of ripening suggests that the resident bacteria in the cheese are probably carrying out deamination reactions and proteolytic degradation of the peptides (Diezhandino *et al*. 2015; Carafa *et al*. 2019).

On the other hand, statistically significant differences ($P \leq 0.001$) were observed in the titratable acidity (TA) throughout the ripening (Figure 2d) and between the values of the different batches and sampling points ($P \leq 0.05$). TA increased markedly during the first 30 days of ripening and to a lesser extent up to 90 days. Thereafter, TA values remained constant until the end of ripening. A significant negative correlation was found between acidity and Aw values ($\rho = -0.40$; $P \leq 0.01$). This correlation means that the rate of acid production, and therefore the metabolic activity of the microorganisms, slows down as Aw decreases. Among the different cheese batches made with the different starter cultures, L4 reported the lowest TA values throughout ripening. In this batch, TA values remained practically stable after 90 days of ripening. After 240 days, all the batches presented TA values higher than 3 g lactic acid 100/g TS, except L4, whose final TA value was 2.89 g lactic acid 100/g TS.

Aw values (Figure 2e) decreased as ripening progressed ($P < 0.001$), from 0.972 ± 0.004 to 0.930 ± 0.002 at the end of the ripening period. This decrease in Aw values was negatively correlated with the percentage of TS ($\rho = -0.82$; $P \leq 0.01$). At the end of ripening, the Aw values recorded were slightly higher than those found by other authors in long-ripened sheep's milk cheeses (Renes *et al*. 2019; Mezo-Solís *et al*. 2020). The values obtained for the salt/moisture ratio in the different cheese batches were similar between batches ($P > 0.05$) at the different sampling points (Figure 2f). The salt/moisture ratio increased significantly ($P < 0.001$) during ripening time due to moisture loss ($\rho = -0.90$; $P \leq 0.01$) in the cheeses, reaching values between 5.58% and 5.95%. Increasing salt/moisture values

Figure 2 Changes in (a) protein/total solids contents, (b) fat/total solids contents, (c) pH, (d) titratable acidity, (e) water activity and (f) salt/moisture values throughout the ripening of Castellano cheese made with different starter and non-starter lactic acid bacteria cultures. Batches of cheese were made with: *T* (●), commercial starter culture; L1 (□), commercial culture + *Lactiplantibacillus plantarum* TAUL67; L2 (♦), commercial culture + *Lactiplantibacillus plantarum* TAUL67 + *Lacticaseibacillus paracasei* TAUL1752; L3 (■), commercial LAB starter culture + *Leuconostoc mesenteroides* TAUL1342 + *Lactiplantibacillus plantarum* TAUL67; L4 (○) *Lactococcus lactis* GE44 + *Leuconostoc mesenteroides* TAUL1342 + *Lactiplantibacillus plantarum* TAUL67. Data are the mean of the two replicates from each batch and each sample was one cheese from each batch and sampling point. Statistically significant differences between batches for each ripening time are expressed as: (*) $P \leq 0.05$, (**) $P \leq 0.01$ and (***) $P \leq 0.001$.

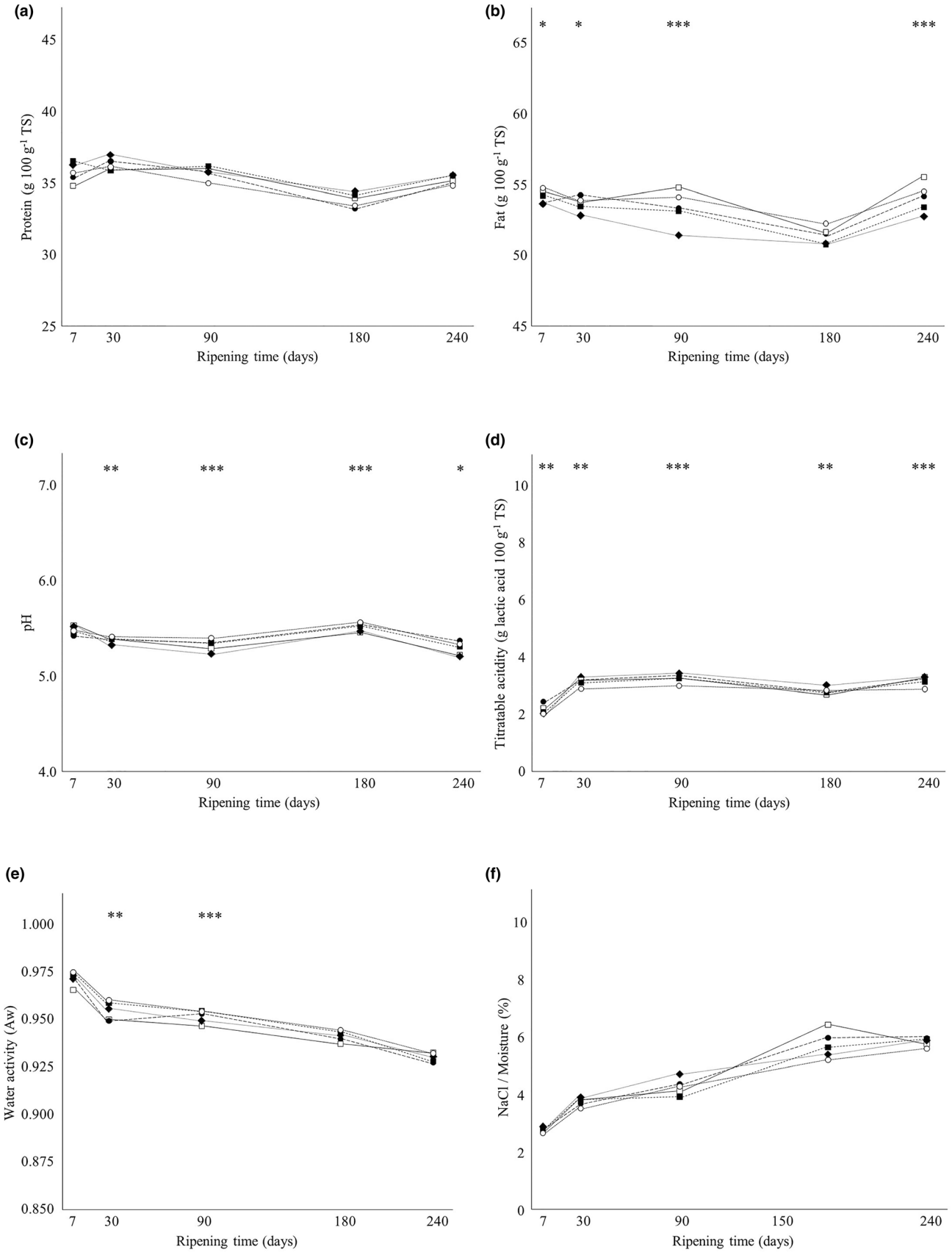

Table 1 Total solids and colour[1] measurement throughout the ripening for the Castellano cheese batches made with different starter and non-starter lactic acid bacteria cultures.

		Ripening time (days)					*P*-value[3]	
Parameter	Batch[2]	7	30	90	180	240	SC[4]	RT[5]
Total solids	*T*	59.62 ± 0.93	60.99 ± 1.26	65.67 ± 1.43	72.96 ± 1.49	71.16 ± 1.44	NS	***
(g 100/g cheese)	L1	60.16 ± 0.82	61.02 ± 1.21	66.95 ± 2.19	74.64 ± 1.22	71.83 ± 0.82		
	L2	60.36 ± 0.79	61.90 ± 0.56	66.71 ± 2.94	72.41 ± 1.70	72.46 ± 2.87		
	L3	58.62 ± 0.80	60.02 ± 1.29	65.31 ± 1.57	72.13 ± 1.37	70.96 ± 1.60		
	L4	59.76 ± 0.48	61.03 ± 0.73	65.75 ± 1.27	71.12 ± 1.18	71.18 ± 2.07		
*L**	*T*	88.10 ± 1.28^{b}	85.94 ± 0.52^{ab}	84.64 ± 0.49^{b}	83.25 ± 1.01	82.39 ± 1.15	NS	***
	L1	86.45 ± 1.48^{ab}	85.51 ± 0.64^{ab}	84.88 ± 1.65^{b}	81.68 ± 2.36	80.89 ± 1.52		
	L2	85.71 ± 1.32^{a}	84.64 ± 1.00^{ab}	83.81 ± 1.06^{ab}	81.50 ± 2.03	81.08 ± 0.41		
	L3	86.51 ± 1.30^{ab}	86.35 ± 0.43^{b}	83.82 ± 0.45^{ab}	82.59 ± 1.53	81.52 ± 1.07		
	L4	87.06 ± 1.05^{ab}	84.01 ± 2.51^{a}	82.49 ± 1.20^{a}	82.27 ± 1.70	79.85 ± 2.20		
*a**	*T*	−2.59 ± 0.14^{a}	−2.37 ± 0.11^{a}	−2.28 ± 0.22	−2.05 ± 0.16	−1.86 ± 0.20	**	***
	L1	−2.38 ± 0.13^{ab}	−2.24 ± 0.13^{ab}	−1.92 ± 0.15	−1.78 ± 0.36	−1.73 ± 0.34		
	L2	−2.31 ± 0.20^{ab}	−1.94 ± 0.14^{b}	−1.92 ± 0.27	−1.82 ± 0.14	−1.83 ± 0.04		
	L3	−2.48 ± 0.35^{ab}	−2.10 ± 0.19^{ab}	−2.04 ± 0.44	−1.87 ± 0.12	−1.78 ± 0.14		
	L4	−2.16 ± 0.22^{b}	−1.96 ± 0.32^{b}	−1.95 ± 0.33	−1.78 ± 0.23	−1.75 ± 0.20		
*b**	*T*	14.41 ± 0.34^{b}	14.66 ± 0.53	14.96 ± 0.76	15.12 ± 0.10	15.62 ± 0.59	***	***
	L1	13.11 ± 0.32^{a}	13.45 ± 0.75	14.95 ± 0.74	14.94 ± 0.45	15.58 ± 1.23		
	L2	13.35 ± 0.59^{a}	14.19 ± 1.61	14.71 ± 1.31	14.94 ± 1.31	15.24 ± 0.42		
	L3	13.15 ± 0.50^{a}	14.51 ± 0.66	14.54 ± 1.20	14.79 ± 1.01	14.87 ± 1.07		
	L4	13.25 ± 0.80^{a}	13.57 ± 0.32	13.76 ± 0.54	13.95 ± 0.67	14.34 ± 0.37		

Means ± SD within the same column with different superscripts alphabets (differences between cheese batches for the same ripening time point) is significantly different ($P \leq 0.05$).

[1]Colour measures *L**, *a** and *b** were determined according to the CIELab colour space, where *L** corresponds to light–dark chromaticity (0% = dark to 100% = light), *a** corresponds to green–red chromaticity (−60% = green to 60% = red) and *b** corresponds to blue–yellow chromaticity (−60% = blue to 60% = yellow).

[2]Batch: T, commercial starter culture; L1, commercial culture + *Lactiplantibacillus plantarum* TAUL67; L2, commercial culture + *Lactiplantibacillus plantarum* TAUL67 + *Lacticaseibacillus paracasei* TAUL1752; L3, commercial starter culture + *Leuconostoc mesenteroides* TAUL1342 + *Lactiplantibacillus plantarum* TAUL67; L4 *Lactococcus lactis* GE44 + *Leuconostoc mesenteroides* TAUL1342 + *Lactiplantibacillus plantarum* TAUL67.

[3]* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; NS = $P > 0.05$.

[4]SC, starter culture effect.

[5]RT, ripening time effect.

were also related to decreasing Aw values ($\rho = -0.76$; $P \leq 0.01$). Salt directly determines the water activity in cheese and therefore affects microbial growth, enzyme activity and biochemical reactions that occur during cheese ripening (Renes *et al*. 2019).

Changes in colour parameters during ripening

Figure 3 shows the colour development of the five batches of cheese during ripening. It can be seen that the colour evolved from white after 7 days to more yellowish shades. In general, the evolution was similar, except for batch T, where a darker colour was observed at the end of ripening than in the other batches.

The colour characteristics analysed had statistically significant effect ($P \leq 0.001$) on *L**, *a** and *b** values with the ripening time; while the starter culture of cheese making had statistically significant effect ($P \leq 0.01$) on *a** and *b** values. Significant negative correlations were found between ripening time and *L** ($\rho = -0.81$; $P \leq 0.01$), *a** ($\rho = -0.65$; $P \leq 0.01$) and *b** ($\rho = -0.22$; $P \leq 0.01$) variables. The decrease in lightness (*L**), the most correlated variable, between 7 and 240 days, was possibly due to protein dehydration leading to a reduction in light scattering. These phenomena are also associated with moisture loss, pH decrease and advancing proteolysis (Ruvalcaba-Gómez *et al*. 2022; Kaczyński *et al*. 2023). On the other hand, a slight increase in redness (*a**) and yellowness (*b**) was observed. Tomar (2019) described that the increase in *a** scores was directly associated with the bacterial strains used as starter cultures. On the other hand, the more intense

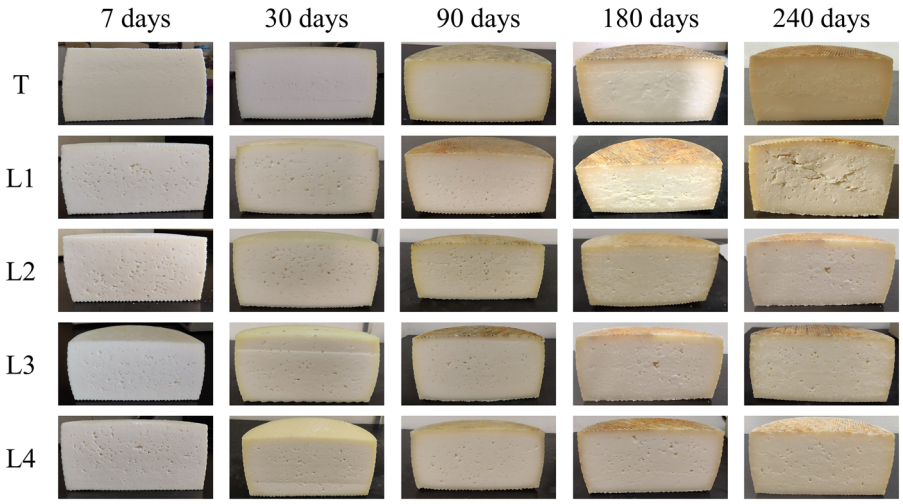

Figure 3 Colour evolution of the five batches of Castellano cheese made with different starter and non-starter lactic acid bacteria cultures throughout the ripening process.

yellow colour of long-ripened cheeses has been linked to the loss of moisture during ripening and the consequent increase in fat content, as well as to oxidative reactions and a higher degree of proteolysis (Bancalari *et al*. 2020; Sharma *et al*. 2020).

Changes in microbial populations during ripening

The values of microbial counts of sheep's milk cheeses at the different ripening times are shown in Figure 4. *Enterobacteriaceae* and enterococci counts, as well as mould and yeast counts, were not detected or were very low (<30 colonies per plate). In the case of moulds and yeast counts, although counts were detected after 90 days of ripening, they were very low in all samples (<20 colonies per plate) and were therefore not taken into account. The absence of *Enterobacteriaceae* and enterococci counts indicated the good sanitary quality of the cheese batches, while the absence of moulds and yeasts could be due to the effect of the application of plastic resins with antifungal compounds on the cheese surface (Renes *et al*. 2021).

The changes in aerobic mesophilic microbiota counts throughout the ripening of the cheeses are shown in Figure 4 (a). At each sampling point, significant differences ($P \leq 0.05$) were found between cheese batches. The PCA counts showed a strong correlation with the physicochemical variables Aw ($\rho = 0.855$; $P \leq 0.01$) and NaCl/humidity ($\rho = -0.79$; $P \leq 0.01$) which would explain why the highest PCA counts were observed in the first week of ripening and decreased during ripening. At the end of ripening, the differences found were statistically highly significant ($P \leq 0.001$). After 240 days of ripening, batch *T* had the lowest counts (5.0 ± 0.4 log ufc/g), while L2 reported counts almost 3 log units higher than *T* (7.8 ± 0.3 log ufc/g).

Counts on M17 (presumptive lactococci) and MSE (presumptive *Leuconostoc*) (Figure 4b,c) presented similar behaviour and values throughout the ripening. Statistically significant differences ($P \leq 0.001$) were found in both counts due to ripening time. The highest counts were obtained in the samples with the shortest maturation time (8 log units after 7 days of maturation) and then decreased to values around 6 log units. The highest counts in M17 at the end of ripening were registered by L4 and *T* (5.9 ± 0.51 and 5.6 ± 0.4 log CFU/g respectively), while L1 had the lowest counts (4.2 ± 0.2 log CFU/g). There was a significant negative correlation between salt/moisture ratio and M17 counts ($\rho = -0.88$; $P \leq 0.01$) and a positive correlation with Aw ($\rho = 0.84$; $P \leq 0.01$). Therefore, the decrease of lactococci during cheese ripening could be influenced by the Aw and the salt/moisture ratio of the cheese as well as by the acidity. The batches with higher counts at M17 had lower titratable acidity and higher pH values. Under conditions of higher acidity, lactococci may be replaced by other more acid-tolerant species. In addition, the lactobacilli population is affected by the hydrolytic activity of their enzymes and competition from other microbial groups better adapted to the adverse conditions (Diezhandino *et al*. 2015). In the case of the MSE counts, there were differences due to the effect of culture ($P \leq 0.001$). MSE counts were higher at the beginning of ripening in batch L3 (9.1 ± 0.1 log CFU/g), which incorporated *Ln. mesenteroides* in the starter culture, but not in L4, which also incorporated *Ln. mesenteroides* (7.7 ± 0.3 log CFU/g). As ripening progressed, the counts decreased in all batches, although this was more pronounced in batches L3 and L4. It should be noted that although it is considered an optional medium for *Leuconostoc* counts, this medium allows for the counting of the lactic acid bacterial population that predominates at the time of ripening. The variables Aw and NaCl/moisture were correlated with MSE counts but the correlation was lower than in the previous cases (Aw, $\rho = 0.47$; $P \leq 0.01$; and NaCl/

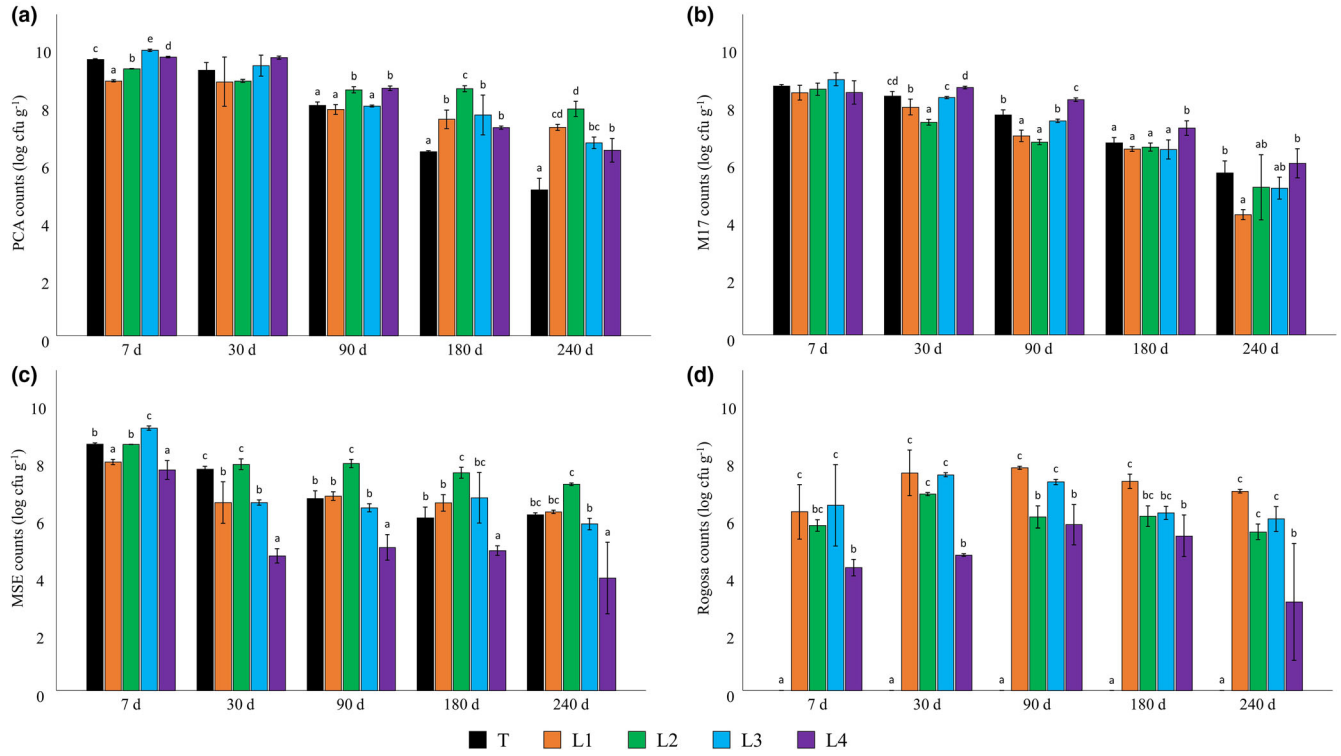

Figure 4 Counts (log CFU/g) of (a) PCA, (b) M17, (c) MSE and (d) Rogosa at 7, 30, 90, 180 and 240 days of ripening of Castellano cheese made with different starter and non-starter lactic acid bacteria cultures. Data are means ± SD of two analyses from each sample and each sample was one cheese from each batch and sampling point. Different letters (a–e) in the same batch at the same ripening time point denote significant statistical differences ($P \leq 0.05$).

moisture, $\rho = -0.48$; $P \leq 0.01$). It was reported that salt concentration did not seem to affect the growth of *Leuconostoc* strains but low pH had a greater effect on the growth of these strains (Özer and Kesenkaş 2019).

Rogosa counts (Figure 4d) increased during the first month of ripening and then remained stable. There were no significant differences due to the effect of ripening time ($P > 0.05$). However, differences were observed due to the effect of culture ($P < 0.001$). It should be noted that there were no counts in batch T, which was prepared with lactococcus strains only. This could explain the low PCA counts obtained in that batch at the end of ripening. On the other hand, batch L4 had significantly lower counts ($P < 0.05$) than the other batches. Lower TA values were observed in batch L4 compared to the rest of the batches. The counts obtained in M17 for batch L4 did not decrease as in the other batches, which could be due to the growth of the lactobacilli population, which was lower than in the other batches. On the other hand, in the batches where acidification was greater, the counts obtained in M17 decreased more as ripening progressed, while the counts in Rogosa increased or remained more stable. A possible inhibitory effect of the *Lc. lactis* GE44 strain on the *L. plantarum* TAUL67 strain could be the explanation for the low counts at all stages of ripening. Finally, between batches L1, L2 and L3 there were no significant differences ($P > 0.05$) at the end of ripening, with counts of 6.9 ± 0.1, 5.5 ± 0.3 and 6.0 ± 0.4 log ufc/g respectively. Lactobacilli are tolerant to the hostile conditions of high salt concentration, high acidity, low Aw and low humidity of the cheese during ripening and can grow when conditions become adverse for the growth of lactococci and *Leuconostoc* (Diezhandino *et al*. 2015). The inclusion of an NSLAB, consisting of strains of lactobacilli with proteolytic and peptidase activity, plays a key role in the sensory properties, as the concentrations of small peptides and free amino acids increase during ripening. (Santamarina-García *et al*. 2022).

Sensory evaluation

The sensory characteristics of cheese are the result of a combination of several factors, including physicochemical and microbiological factors directly related to the milk and the technological process (Chessa *et al*. 2021). During cheese ripening, LAB, particularly NSLAB, modify milk constituents through complex anabolic and catabolic systems. These modifications improve the quality and complexity of the flavour profile (Mayo *et al*. 2021). Figure 5 shows the FAEPC of the sensory variables analysed

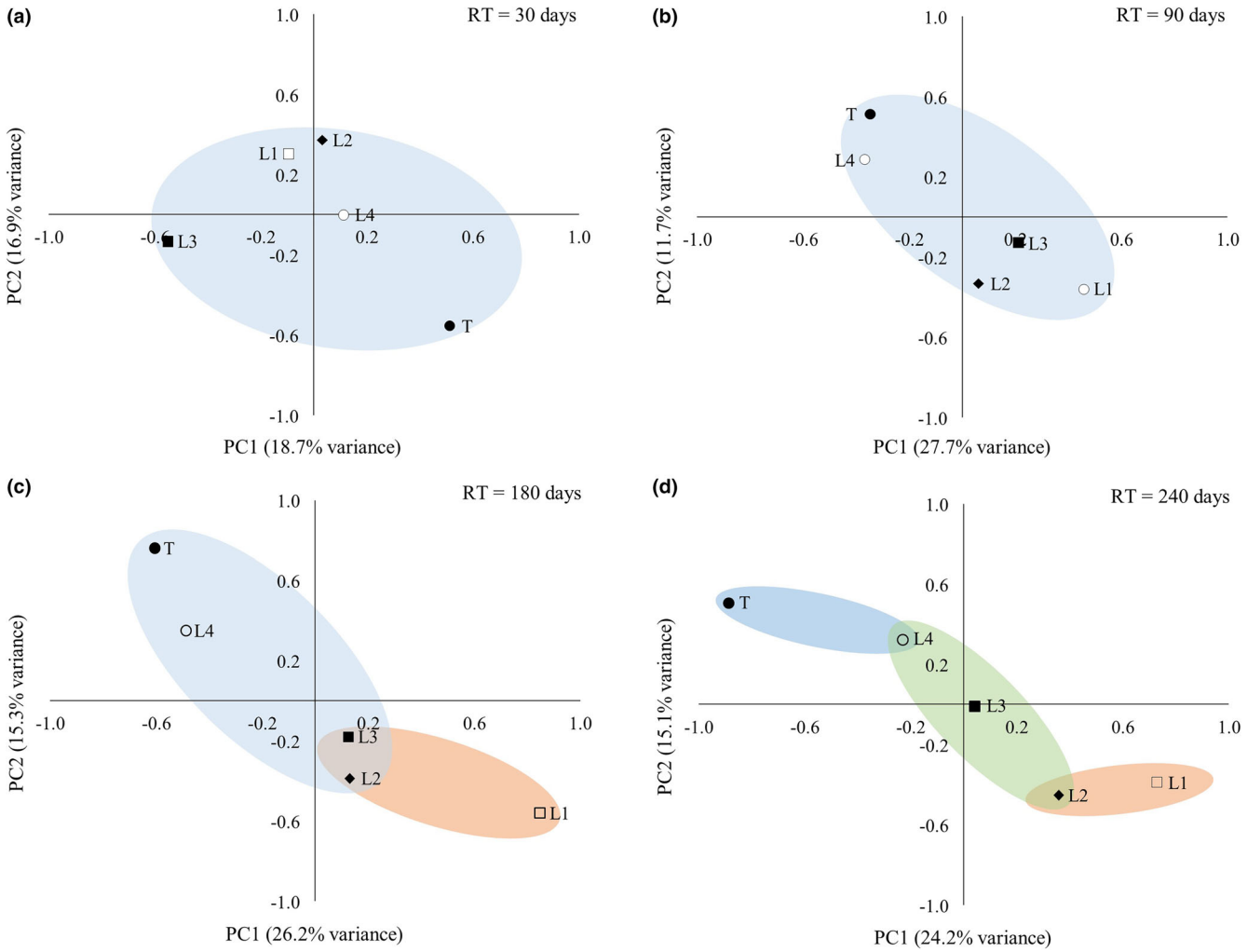

Figure 5 Factor analysis with principal component extraction (FAEPC) of the sensory variables analysed in the five batches of Castellano cheese after 30, 90, 12 and 240 days of ripening. Different coloured circles in the same batch at the same ripening time denote significant statistical differences ($P \leq 0.05$). PC1, principal component 1; PC2, principal component 2; RT, ripening time.

(appearance, odour, taste and texture) in the five batches of Castellano cheese during the ripening time. After 30 days of ripening (Figure 5a), there were no significant differences ($P > 0.05$) in the values of the principal components (PC) 1 and 2; similar to what was observed after 90 days of ripening (Figure 5b). However, after 180 days, statistically significant differences were observed ($P \leq 0.05$). As shown in Figure 5c, batches *T* and L4 are completely separated from L1. Finally, after 240 days of ripening (Figure 5d), three groups of significance ($P \leq 0.05$) appear: the first one consisting of *T* and L4 batches, the second one including L1 and L2 and a third one where L3 is found, which not showed differences with L2 and L4. In view of these results, it seems that the differences between cheese batches are due to the effect of lactobacilli growth during ripening, as also pointed out by other authors (Blaya *et al*. 2018).

Table 2 shows the average scores awarded by the panel of tasters for various sensory attributes (appearance, aroma, flavour and texture) of the five batches of Castellano cheese produced with different starter and non-starter cultures after 240 days of ripening. In addition, the average result of the overall evaluation of each cheese batch is shown. Appearance attributes showed significant differences ($P \leq 0.001$) between the cheeses. The occurrence of internal cracks in some cheese batches was significant in the later stages of ripening (180 and 240 days). Cracking was particularly observed in cheese batch L1 and to a lesser extent in L2 and L3. The formation of internal cracks can also be attributed to microbial activity (Serrapica *et al*. 2020). In Figure 4 (d), it can be seen how lactobacilli counts were higher in L1, as well as, in L2 and L3. The production of lactic acid by lactobacilli in late ripening stages (Figure 2b) could be related to the appearance of internal cracks at the end of

Table 2 Average values ± SD assigned by tasting panel for various sensory attributes linked to appearance, aroma, texture and flavour, and an overall evaluation for ewe's milk cheese made with different starter cultures after 240 days of ripening.

	Batch[1]					
Attribute[2]	*T*	L1	L2	L3	L4	*P*-value[4]
Appearance						
Colour intensity	3.86 ± 0.77	3.93 ± 0.83	4.07 ± 0.73	3.79 ± 0.58	3.86 ± 0.86	NS
Cracks	1.50 ± 0.65^{a}	4.14 ± 1.79^{b}	2.93 ± 1.73^{ab}	2.29 ± 1.14^{ab}	1.43 ± 0.51^{a}	***
Holes number	1.71 ± 0.61^{a}	4.43 ± 1.22^{b}	4.71 ± 0.91^{b}	4.07 ± 1.00^{b}	3.43 ± 0.76^{ab}	***
Holes size	1.71 ± 0.47^{a}	4.14 ± 0.77^{c}	3.71 ± 0.73^{bc}	3.14 ± 0.77^{bc}	3.00 ± 0.68^{b}	***
Holes homogeneity	2.14 ± 0.95^{a}	3.50 ± 1.40^{ab}	3.86 ± 1.17^{b}	3.71 ± 0.99^{b}	3.93 ± 1.33^{b}	**
Crystals	1.14 ± 0.36^{a}	2.71 ± 1.14^{bc}	4.07 ± 1.27^{c}	2.79 ± 0.80^{c}	1.57 ± 0.51^{ab}	***
Odour						
Odour intensity	3.57 ± 0.85	4.43 ± 1.22	3.93 ± 0.62	3.93 ± 1.07	4.00 ± 0.55	NS
Odour persistence	3.00 ± 0.68	3.86 ± 1.23	3.29 ± 0.99	3.64 ± 1.01	3.64 ± 0.74	NS
Rancid, butyric	1.50 ± 0.76	2.36 ± 1.15	2.50 ± 1.40	2.21 ± 1.37	2.00 ± 1.30	NS
Spicy, acetic, ammonia	1.36 ± 0.63^{a}	3.57 ± 1.79^{b}	2.64 ± 1.74^{ab}	2.79 ± 1.37^{ab}	2.43 ± 1.34^{ab}	**
Soap, sulphurous	1.29 ± 0.47	2.29 ± 1.54	1.93 ± 1.00	1.79 ± 1.19	1.71 ± 0.99	NS
Mouldy	1.21 ± 0.43	1.64 ± 0.84	1.36 ± 0.63	1.29 ± 0.47	1.36 ± 0.63	NS
Taste						
Taste intensity	4.21 ± 1.05	5.14 ± 1.10	4.57 ± 0.51	4.50 ± 0.65	4.50 ± 1.02	NS
Taste persistence	3.71 ± 1.14^{a}	4.93 ± 0.83^{b}	4.43 ± 1.09^{ab}	4.00 ± 0.68^{ab}	4.36 ± 0.93^{ab}	*
Sweet	1.93 ± 1.00	1.79 ± 1.19	1.86 ± 1.17	2.43 ± 1.45	2.14 ± 1.29	NS
Acid	3.86 ± 1.03	4.50 ± 1.02	4.00 ± 1.30	3.86 ± 1.29	4.21 ± 1.25	NS
Salty	3.71 ± 0.73	4.07 ± 1.07	3.57 ± 0.94	3.64 ± 0.93	3.64 ± 1.08	NS
Bitter	3.57 ± 1.16	3.21 ± 1.37	3.07 ± 1.27	3.07 ± 1.27	2.93 ± 1.27	NS
Texture						
Elasticity	2.36 ± 0.93^{ab}	1.86 ± 1.10^{a}	1.79 ± 1.05^{a}	2.57 ± 1.02^{ab}	3.14 ± 0.95^{b}	**
Firmness	4.29 ± 0.91	4.86 ± 1.23	4.86 ± 0.95	4.29 ± 0.91	3.93 ± 0.73	NS
Friability	3.14 ± 1.17^{a}	5.57 ± 1.16^{b}	4.50 ± 1.65^{ab}	4.00 ± 1.30^{ab}	3.00 ± 1.11^{a}	***
Adhesiveness	2.57 ± 1.02	2.21 ± 1.19	2.29 ± 1.33	2.50 ± 1.02	2.86 ± 1.03	NS
Solubility	2.79 ± 1.05	2.50 ± 1.02	2.43 ± 0.85	2.64 ± 0.63	3.29 ± 0.73	NS
Creaminess	3.14 ± 1.17	2.50 ± 1.22	2.50 ± 1.16	2.93 ± 0.92	3.43 ± 1.16	NS
Overall evaluation						
Overall score[3]	5.50 ± 0.65	4.93 ± 1.86	4.86 ± 1.70	5.71 ± 1.44	5.79 ± 1.72	NS

Means ± SD within a row with different superscripts alphabets are significantly different ($P \leq 0.05$).

[1]T, commercial starter culture; L1, commercial culture + *Lactiplantibacillus plantarum* TAUL67; L2, commercial culture + *Lactiplantibacillus plantarum* TAUL67 + *Lacticaseibacillus paracasei* TAUL1752; L3, commercial starter culture + *Leuconostoc mesenteroides* TAUL1342 + *Lactiplantibacillus plantarum* TAUL67; L4 *Lactococcus lactis* GE44 + *Leuconostoc mesenteroides* TAUL1342 + *Lactiplantibacillus plantarum* TAUL67.

[2]All attributes were scored between 1 and 7.

[3]Overall evaluations were scored between 1 and 10.

[4]* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$; NS = $P > 0.05$.

ripening. On the other hand, both the presence of holes in the cheese and their distribution and size showed significant differences ($P < 0.001$). Batch *T* had very few holes, while the other batches had many. Although there was a positive correlation between the number of holes and the Rogosa counts ($\rho = 0.55$; $P \leq 0.01$), it is not possible to conclude that these holes in the cheese mass are due to the effects of the culture or simply a consequence of the ripening process. Finally, significant differences ($P < 0.001$) were found between cheese batches due to the presence of crystals. The presence of crystals was particularly noticeable in batch L2, and to a lesser extent in batches L1 and L3. Residual lactose is mainly converted to L-lactate by SLAB. In contrast, the less soluble D-lactate is produced when NSLAB reach high populations (mainly pediococci and mesophilic lactobacilli) by fermentation of residual lactose or by isomerisation of L-lactate (Blaya *et al.* 2018). Odour attributes showed no significant differences between batches ($P > 0.05$), except

for the odour descriptor spicy, acetic and ammonia ($P \leq 0.01$). In general, the odour attributes scored higher in the batches that included NSLAB, with batch L1 scoring the highest in all attributes. There were positive correlations between lactobacilli counts and the attributes of odour intensity ($\rho = 0.35$; $P \leq 0.01$) and persistence ($\rho = 0.33$; $P \leq 0.01$). With regard to taste attributes, positive correlations were found between lactobacilli counts and the attributes of flavour intensity ($\rho = 0.31$; $P \leq 0.01$) and persistence ($\rho = 0.31$; $P \leq 0.01$). There were only significant differences ($P \leq 0.05$) in the persistence of flavour. Batch *T* scored the lowest (3.71 ± 1.14) while L1 scored the highest (4.93 ± 0.83). It was also observed that cheeses containing lactobacilli had some flavour defects linked to excessive acidity, with a very sour aftertaste, a phenomenon previously reported by Herreros *et al*. (2007). Finally, texture attributes showed no significant differences between batches ($P > 0.05$), except for elasticity ($P \leq 0.01$) and friability ($P \leq 0.001$). In the case of elasticity, cheese batch L4 scored the highest (3.14 ± 0.95), while L2 was the least elastic (1.79 ± 1.05). The highest friability was obtained by cheese batch L1 (5.57 ± 1.16), while the least friable cheese was L4 (3.00 ± 1.11). NSLABs are involved in proteolysis and lipolysis during cheese ripening. These processes result in an increased concentration of small peptides, free amino acids and free fatty acids during ripening, which accelerates ripening and contributes to the development of flavour and texture (Carafa *et al*. 2019; Santamarina-García *et al*. 2022).

CONCLUSION

The use of different autochthonous strains, especially NSLAB strains, in the production of PGI Castellano cheese influenced the changes in the microbiota and in the physicochemical parameters, especially on pH and titratable acidity, as well as in the sensory characteristics of the Castellano cheese made with pasteurised milk. Incorporation of NSLAB strains gave cheeses made with pasteurised milk similar or better flavour and aroma characteristics than the batch made with commercial starter alone. This could allow a reduction in ripening time without compromising sensory characteristics.

These results will help to increase knowledge of the evolution of the physicochemical, microbiological and sensory characteristics of PGI Castellano cheese during ripening. In addition, this study provides information on the potential use of autochthonous strains in the production of PGI Castellano cheese and their impact on the quality of the final product, which can be extended to other types of cheese. The results of this study could be useful for producers of PGI Castellano cheese who wish to improve the quality of their products and reduce the ripening time, which is of great industrial and, consequently, economic interest.

ACKNOWLEDGEMENTS

The authors thank the University of León (León, Spain) for granting a PhD fellowship to Daniel Abarquero. In addition, the authors wish to acknowledge the collaboration and advice of "Queserías Entrepinares" (Valladolid, Spain) in the manufacture, ripening and sensory analysis of the cheeses used in this study.

AUTHOR CONTRIBUTIONS

Daniel Abarquero: Conceptualization; formal analysis; investigation; methodology; validation; writing – original draft. **Raquel Bodelón:** Methodology. **Catalina Manso:** Methodology. **Pablo Rivero:** Methodology. **José María Fresno:** Conceptualization; methodology; writing – review and editing. **María Eugenia Tornadijo:** Conceptualization; supervision; writing – review and editing.

CONFLICT OF 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.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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