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Production of sheep milk cheese with high γ-aminobutyric acid and ornithine concentration and with reduced biogenic amines level using autochthonous lactic acid bacteria strains

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ABSTRACT

Consumer demand for health-promoting foods is generating the need to develop biofunctional dairy products. Lactic acid bacteria are employed in cheese-making and some of them are able to produce beneficial compounds on human health such as γ-aminobutyric acid (GABA) and ornithine but also to synthetize biogenic amines. The aim was to investigate the effect of four selected autochthonous co-cultures on the free amino acid profile, with special emphasis on GABA and ornithine, and on the biogenic amine content of pasteurized sheep milk cheese during ripening. High average concentrations of GABA (1296.75 mg/kg cheese) and ornithine (2355.76 mg/kg cheese) were found in all the cheese batches at 240 days of ripening. Batch 2, manufactured with the co-culture containing autochthonous *Lactococcus lactis* strains as starter and *Lactobacillus plantarum* TAUL1588 as adjunct, showed 2.37 fold reduced biogenic amines concentration with respect to the batch 1 made with the starter during the ripening time. The microstructure and microbiological counts of cheeses were affected ($P \leq 0.001$) by the ripening time, without appreciating differences ($P \geq 0.05$) in the physico-chemical composition between batches. This study could be a good approach to the development of functional sheep milk cheese.

1. Introduction

The relationship between food and consumer health has become a priority concern in food production. Balthazar et al. (2017) have stated that sheep milk, which is mainly used for cheese production, is an excellent source of nutrients. At present, the hygienic quality of cheese is guaranteed by the pasteurization of milk. This thermal treatment causes the elimination of part of the milk's microbiota and this fact makes necessary the use starters and adjunct cultures to guaranteeing the appropriate sensory characteristics of each cheese variety (Minervini et al., 2009). Lactic acid bacteria (LAB) are usually employed as cultures for cheese-making and play a very important role in the proteolysis that takes place during cheese ripening since they contain proteinases and peptidases that can lead to the production of free amino acids (Fox et al., 2016). Several studies have been carried out to study the effect of different autochthonous LAB strains on the content of free amino acids in cheese (Madrau et al., 2006; Mangia et al., 2008; Poveda et al., 2015, 2004). These free amino acids can act as substrates for secondary catabolic reactions by LAB, leading to the formation of compounds such as gamma-aminobutyric acid (GABA) and ornithine (Manca et al., 2015). These two compounds have recently attracted the attention of the food industry since GABA and ornithine have numerous beneficial physiological functions on human health (Adeghate and Ponery, 2002; Diana et al., 2014a; Sugino et al., 2008).

It has been observed that various strains of LAB, such as *Lactobacillus brevis* DPC6108, *Lb. brevis* PM17, *Lactobacillus plantarum* C48, *Lactobacillus paracasei* PF6 and *Lactococcus lactis* PU1, were able to synthesize GABA when they are grown in culture medium supplemented with monosodium glutamate (Barrett et al., 2012; Siragusa et al., 2007). It has been also observed that the capacity to synthesize GABA by LAB is dependent on the strain and not on the specie (Dhakal et al., 2012). Several studies have been carried out in order to know the concentration of this bioactive compound in commercial cheeses (Diana et al., 2014b; Manca et al., 2015; Poveda et al., 2016). However, as Diana et al. (2014b) have indicated little attention has been given to ornithine. Likewise, few studies have been focused on testing the ability of autochthonous LAB cultures to produce GABA and ornithine during cheese making (Poveda et al., 2004; Poveda et al., 2015).

However, it must be taken into account that some decarboxylation reactions can lead to toxic compounds such as biogenic amines that can

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also be synthesized during cheese ripening. Biogenic amines are organic, basic, nitrogenous compounds with biological activity. The consumption of foods containing large amounts of these amines can provoke toxicological effects and these problems can be more severe in consumers in whom detoxification is less efficient because of their genetic constitution or if they are under some treatments (Linares et al., 2011). Cheese is one of the most prevalent foods associated with amine poisoning and the consumption of cheese that has a high level of tyramine may result in a dangerous intoxication characterized by an increase in blood pressure (Ladero et al., 2010). For this reason, when cultures are designed to be employed in the production of cheese with an amino acid profile beneficial on human health, it is also necessary to ensure that these cultures do not generate high concentrations of biogenic amines in the final product.

The objective of this study was to investigate the effect of the use of four different autochthonous co-cultures on the free amino acid profile, with special emphasis on GABA and ornithine, on the biogenic amine content, microstructure, physico-chemical and microbiological parameters of pasteurized sheep milk cheese during ripening.

2. Material and methods

2.1. Preparation of cultures

The autochthonous cultures used in sheep cheese manufacture were selected based on their good technological aptitude showed in previous studies (González et al., 2015; Herreros et al., 2003).

Before culturing, each LAB strain was activated in either MRS broth (Oxoid, Hampshire, UK) for *Lactobacillus* or Elliker (BD Difco, New Jersey, USA) for *Lactococcus lactis*, and then in reconstituted skim milk (10%, w/v) at 30 °C for 24 h. The strain composition of the designed cultures for cheese manufacture is shown in Fig. 1. Finally, each activated autochthonous cultures were transferred at 1% (v/v) to sterilized sheep milk for 48 h at 30 °C.

2.2. Milk and cheese manufacture

Sheep milk was obtained over four consecutive days from a local farm which belongs to the Consortium for Ovine Promotion (Villalpando, Zamora, Castilla-León, Spain).

Four sheep cheese batches were manufactured in duplicate at pilot scale (Institute of Food Science and Technology (ICTAL), University of León, Spain) according to the following method: 75 L of sheep milk (for each batch and replicate) was pasteurized at 72 °C for 15 s and after cooling at 31 °C, calcium chloride (0.2 g/L) and starter culture or starter culture plus adjunct (1%, v/v) were added. The composition of the co-cultures used for each cheese batch is indicated in Fig. 1. After 30 min, chymosin (CHY-MAX Extra, 100% chymosin; 600 IMCU/mL; Chr. Hansen SL, Madrid, Spain) was added at a rate of 0.05 mL/L of milk (diluted in 1:20 with deionized water). After 40–45 min, the curd was cut to rice grain size and the whey was drained off. The curd was transferred to cylindrical molds (15 cm height, 21 cm diameter) which were pressed for 2 h. Then, cheeses were salted by immersion (18°Baume, 8 °C and pH 5.4) for 17 h. Finally, the cheeses without being packaged were taken to a ripening chamber where they remained at a temperature of 10 °C and at 80–85% relative humidity for 240 d.

Samples were taken from each batch after 2, 90, 180 and 240 days of ripening. The samples (each sample corresponded to a whole cheese of 2.5 kg) were grinded, packed and stored in a freezer (−30 °C) until analysis. Microstructure, physico-chemical and microbiological analyses were carried out on fresh samples.

2.3. Physico-chemical analysis

The pH and titratable acidity of cheese batches were determined according to standard 14.022 (AOAC, 1980a, 1980b). Water activity

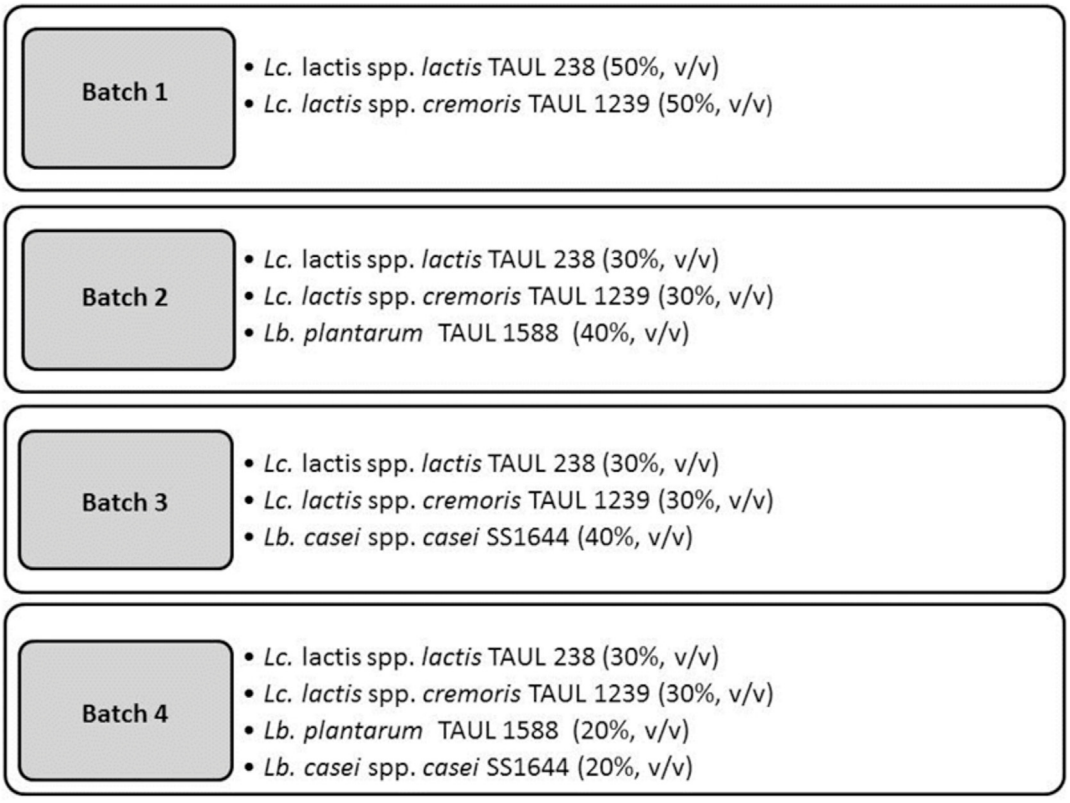

Fig. 1. Strain composition of autochthonous cultures. Volume of each individual strain activated in milk respect to the final volume of the mixed culture in sheep milk.

(A_w) was analyzed instrumentally using an Aqua Lab Dew Point Analyzer CX-2 (Decagon Devices, Pullman, WA, USA). Total solids, NaCl, fat and protein contents were determined according to standards 004 (FIL-IDF, 2004), 935.43 (AOAC, 1990), 221 (FIL-IDF, 2008), 20–1 (FIL-IDF, 2001), respectively. All samples were carried out in duplicate.

2.4. Microbiological analysis

Fifty grams of milk or cheese samples were homogenized with 200 mL of a 2% (w/v) sodium citrate solution (Panreac, Barcelona, Spain) in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Decimal dilutions were prepared by mixing 10 mL of this homogenate with 90 mL of sterile peptone water (Oxoid, Unipath, Ltd., Basingstoke, UK) at 0.1% (w/v) according to standard 122B (FIL-IDF, 1992).

Aerobic mesophilic bacteria were enumerated on standard Plate Count Agar (PCA; Oxoid) after incubation at 30 °C for 48 h. LAB were determined on De Man-Rogosa-Sharpe (MRS) agar (Oxoid) after incubation at 30 °C for 72 h. Lactobacilli were enumerated on Rogosa agar (Oxoid) incubated at 30 °C during 5 days. *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBGA; Oxoid) after incubation at 37 °C for 18–24 h.

2.5. Determination of free amino acids and biogenic amines by ultra high performance liquid chromatography

Free amino acids and biogenic amines were determined following the method described by Redruello et al. (2013). Briefly, amino acids and biogenic amines were extracted by the homogenization of 1 g of cheese in 10 mL of 0.1 M HCl-0.2% 3,3′thiodipropionic acid (Sigma-Aldrich, Madrid, Spain). This mixture was kept in an ultrasonic bath Bransonic 221 (Branson Ultrasonics S.A, Danbury, USA) for 30 min and then centrifuged at 5000 × g for 20 min. The supernatant was deproteinised by passing through ultrafiltration inserts (Amicon Biomax 5 K; Millipore, MA, USA) by centrifugation at 3500 × g for 1 h. 20 μL of this diluted sample were derivatised with diethyl ethoxymethylenemalonate (DEEMM; Sigma-Aldrich). Duplicate samples were freshly derivatised before injection in the chromatograph system. L-2-aminoadipicacid (Sigma-Aldrich) was used as internal standard. The chromatograph system consisted of an H-Class Acquity UPLC™ system (Waters, Milford, MA, USA) coupled to a photodiode array detector. Free amino acids and biogenic amines separation was carried out using a Waters Acquity UPLC™ BEH C18 column (1.7 μm particle size, 100 mm × 2.1 mm I.D.) held at 35 °C. The mobile phase consisted of 25 mM acetate buffer pH 6.7 plus 0.02% sodium azide (eluent A), methanol (eluent B) and acetonitrile (eluent C). Samples (1 μL) were applied to the column and eluted at a flow rate of 0.45 mL/min according to the linear gradient used by Redruello et al. (2013). The target compounds were identified by their retention times and their spectral characteristics at 280 nm, and were quantified using the internal standard method. Data were acquired and analyzed using the software Empower 2 (Waters, Milford, MA, USA).

2.6. Confocal scanning laser microscopy (CSLM)

The four sheep cheese batches were examined using CSLM at 2, 90, 180 and 240 days of ripening, as described by Auty et al. (2001), in order to visualize the changes that took place in the distributions and microstructures of the fat and protein. Cheese sample measuring 10 mm × 10 mm × 5 mm was stained with 50 L of a probe mixture constituted by Nile Red (Sigma-Aldrich) 0.02 g/L and Fast Green FCF (Sigma-Aldrich) 0.1 g/L and examined using a Zeiss LSM310 confocal laser scanning microscope (Carl Zeiss, Welwyn Garden City, Herts, UK). Dual excitation using 488 nm/633 nm for Nile Red/Fast Green FCF was used.

2.7. Statistical analysis

Statistical analysis of the experimental data was performed using SPSS v.21 (SPSS, Chicago, IL, USA). The free amino acids, biogenic amines, physico-chemical and microbiological variables were tested for the assumption of normality using the Shapiro-Wilk test and for homoscedasticity using the Levene test. Subsequently, a two-way Analysis of Variance (ANOVA) was performed in order to evaluate the effect of the factors co-culture and ripening time (as fixed factors) and the interaction between them. Tukey's HSD *post hoc* test was applied at a 5% significance level in order to compare sheep cheeses manufactured with different autochthonous cultures throughout the different ripening times. The Spearman's rank correlation coefficient (ρ) was applied to estimate the relationship between the physico-chemical parameters and microbiological populations of cheese samples.

3. Results and discussion

3.1. Physico-chemical composition of sheep milk cheeses during ripening

The changes in the physico-chemical parameters of the four sheep milk cheeses throughout ripening are shown in Table 1. These results evidenced that the different co-cultures used for cheese-making did not have a statistically significant effect ($P < 0.05$) on most of the analyzed parameters at the same ripening time. Nevertheless, the titratable acidity values for the 3 and 4 batches manufactured with co-cultures containing the *Lactobacillus casei* subsp. *casei* SS1644 were higher than the other two cheese batches at 90 and 180 days of ripening. The greater acidifying capacity of this strain with respect to the *Lb. plantarum* TAUL 1588 strain was previously reported in other studies (González et al., 2015; Herreros et al., 2003).

It is well known that during ripening a very complex set of interrelated biochemical processes happen and they are responsible of cheese characteristics (Fox et al., 2016).

The evolution of the physico-chemical parameters analyzed during the ripening time allowed corroborating that the process of cheese-making for the 4 batches of cheese was similar. At the beginning of ripening (2 days), there was an increase in pH until 90 days to subsequently remain constant or decrease slightly. The increase observed in the values of this parameter could be due mainly to the buffer capacity that can present the cheese curd during the ripening process (Salaün et al., 2005). This fact prevents obtaining information on microbial growth, however, a clear indicator of the metabolic activity of the co-cultures used in the cheese-making was the increase of the titratable acidity values until 180 days of ripening to later descend. Moreover, the acidification that took place during the ripening process of the cheese batches contributed to the internal drying of the cheese. A significant negative correlation was found between acidity and moisture values ($\rho = -0.84$; $P \leq 0.01$). Moisture decreased significantly ($P \leq 0.001$) throughout ripening, reaching values lower than 30%. A_w followed a similar trend, the mean values decreased as ripening progressed, from 0.989 to 0.915 at the end of the ripening period. It was observed that as the salt/moisture ratio increased, A_w values decreased ($\rho = -0.87$; $P \leq 0.01$). Guinee (2004) has described that salt is a major determinant of the A_w parameter, and thereby exerts control over microbial growth, enzyme activity, and biochemical changes during cheese ripening. The salt/moisture ratio initially increased up to 180 days of ripening to finally remain constant, reaching a value of around 5% in all cheese batches. These A_w and salt/moisture values were similar to those described by other authors for ripened sheep milk cheeses (Guinee and Fox, 2004).

During the ripening period no significant ($P \geq 0.05$) differences were observed in fat and protein in dry matter contents. The values obtained were comparable with those described by other authors for sheep milk cheese (Fernández et al., 2012).

Table 1
Changes in pH, titratable acidity, water activity (A_W) values and in moisture, salt/moisture, fat and protein contents of four sheep milk cheese batches throughout ripening.

Physico-chemical parameter	Batch[a]	Ripening time (d)				*P*-value[b]		
		2	90	180	240	R	B	R*B
pH	1	5.26 ± 0.03[a]	5.32 ± 0.05[a]	5.33 ± 0.01[a]	5.30 ± 0.02[a]	*	NS	NS
	2	5.26 ± 0.01[a]	5.28 ± 0.02[a]	5.31 ± 0.01[a]	5.30 ± 0.01[a]	*	NS	NS
	3	5.27 ± 0.03[a]	5.34 ± 0.03[a]	5.29 ± 0.01[a]	5.28 ± 0.01[a]	*	NS	NS
	4	5.24 ± 0.02[a]	5.31 ± 0.01[a]	5.29 ± 0.01[a]	5.26 ± 0.01[a]	*	NS	NS
Titratable acidity	1	16.51 ± 1.30[a]	17.50 ± 0.50[a]	22.44 ± 0.52[a]	20.88 ± 0.33[a]	**	*	NS
(g lactic acid/kg total solids)	2	16.90 ± 0.31[a]	17.95 ± 0.15[a]	22.83 ± 0.52[a]	20.45 ± 0.54[a]	***	*	NS
	3	17.01 ± 0.50[a]	20.61 ± 1.45[b]	23.90 ± 0.10[b]	21.17 ± 0.21[a]	**	*	NS
	4	16.19 ± 0.13[a]	20.11 ± 0.67[b]	23.42 ± 0.42[b]	21.31 ± 0.33[a]	***	*	NS
A_W	1	0.990 ± 0.001[a]	0.966 ± 0.001[a]	0.945 ± 0.001[a]	0.913 ± 0.001[a]	***	NS	NS
	2	0.987 ± 0.001[a]	0.968 ± 0.000[a]	0.944 ± 0.001[a]	0.917 ± 0.001[a]	***	NS	NS
	3	0.987 ± 0.001[a]	0.965 ± 0.001[a]	0.938 ± 0.000[a]	0.912 ± 0.001[a]	***	NS	NS
	4	0.991 ± 0.001[a]	0.970 ± 0.001[a]	0.940 ± 0.000[a]	0.918 ± 0.000[a]	***	NS	NS
Moisture	1	411.01 ± 11.70[a]	337.79 ± 4.21[a]	286.15 ± 0.44[a]	281.04 ± 3.52[a]	***	NS	NS
(g/kg cheese)	2	399.55 ± 4.39[a]	341.35 ± 1.71[a]	296.58 ± 1.41[a]	278.46 ± 1.72[a]	***	NS	NS
	3	409.43 ± 8.36[a]	338.87 ± 1.57[a]	288.03 ± 4.89[a]	279.73 ± 0.30[a]	***	NS	NS
	4	399.09 ± 10.61[a]	340.22 ± 2.41[a]	297.30 ± 5.97[a]	280.24 ± 0.30[a]	***	NS	NS
Salt/moisture	1	15.08 ± 0.40[a]	43.38 ± 0.11[a]	54.15 ± 0.39[a]	53.23 ± 0.20[a]	**	NS	NS
(g salt/kg moisture)	2	16.20 ± 0.17[a]	43.42 ± 0.12[a]	54.66 ± 1.20[a]	53.93 ± 0.81[a]	**	NS	NS
	3	15.89 ± 0.33[a]	43.07 ± 0.38[a]	54.02 ± 0.65[a]	53.34 ± 0.10[a]	**	NS	NS
	4	16.11 ± 0.43[a]	43.16 ± 0.17[a]	54.50 ± 0.70[a]	53.87 ± 0.61[a]	**	NS	NS
Fat	1	551.32 ± 3.35[a]	555.20 ± 2.70[a]	555.22 ± 5.06[a]	555.19 ± 2.01[a]	NS	NS	NS
(g/kg total solids)	2	550.52 ± 2.60[a]	554.86 ± 1.14[a]	553.90 ± 6.17[a]	552.97 ± 1.06[a]	NS	NS	NS
	3	550.47 ± 5.37[a]	555.40 ± 1.71 [a]	555.46 ± 5.06[a]	555.47 ± 2.12[a]	NS	NS	NS
	4	549.73 ± 5.35[a]	555.05 ± 2.99[a]	554.21 ± 6.01[a]	554.32 ± 3.35[a]	NS	NS	NS
Protein	1	344.55 ± 3.40[a]	338.23 ± 4.53[a]	347.60 ± 3.54[a]	348.88 ± 3.21[a]	NS	NS	NS
(g/kg total solids)	2	344.47 ± 2.26[a]	338.02 ± 3.41[a]	348.26 ± 4.41[a]	348.96 ± 5.11[a]	NS	NS	NS
	3	345.10 ± 2.38[a]	338.75 ± 3.41[a]	348.51 ± 4.31[a]	349.01 ± 4.35[a]	NS	NS	NS
	4	344.97 ± 2.13[a]	338.33 ± 5.30[a]	348.92 ± 4.16[a]	349.06 ± 5.14[a]	NS	NS	NS

Results expressed as mean values ± standard deviation, n = 4.
[a-b] Different superscript letters in the same column denote significant statistical differences ($P < 0.05$) between cheese batches.
[a] 1: *Lactococcus lactis* subsp. *lactis* TAUL 238 + *Lactococcus lactis* subsp. *cremoris* TAUL 1239; 2: starter 1 + *Lactobacillus plantarum* TAUL 1588; 3: starter 1 + *Lactobacillus casei* subp. *casei* SS 1644; 4: starter 1 + *Lactobacillus plantarum* TAUL 1588 + *Lactobacillus casei* subp. *casei* SS 1644.
[b] R: ripening time fixed effect; B: batch fixed effect; R*B: interaction between the fixed effects. NS $P \geq 0.05$; *$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

3.2. Microbial populations of sheep milk cheeses during ripening

The changes in the microbial counts of the four cheese batches throughout the ripening time are reported in Table 2. The aerobic mesophilic microbiota, lactic acid bacteria and lactobacilli counts increased significantly ($P \leq 0.001$) from the pasteurized milk inoculated with each culture (0 h) until the initial stage of ripening. Diezhandino et al. (2015) indicated that this increase in counts is due to the physical retention of microorganisms in curds and to microbial multiplication during the coagulation phase of cheese-making.

The highest counts for aerobic mesophilic microbiota were observed at 2 days of ripening (10 log CFU/mL), except for batch 3, in which the maximum counts were detected at 90 days (11 log CFU/mL). From this point, aerobic mesophilic bacteria gradually decreased towards the end of the ripening with values ranging 7 (batch 1) and 9 (batch 3) log units.

The lactic acid bacteria counts throughout the ripening were similar to those observed in the mesophilic aerobic microbiota, which reflects that the lactic acid bacteria were the predominant microorganisms in the four sheep cheese batches. There was a significant negative correlation between lactic acid counts and the salt/moisture ratio ($\rho = -0.57$; $P \leq 0.01$). Lactic acid bacteria counts increased until 2 days, after which they decreased approximately 1–2 log CFU/mL until the end of the ripening time. The large increase in salt/moisture ratio values at 90 days (Table 1) could exert an inhibitory effect on the lactic acid bacteria growth.

Lactobacilli are considered as non-starter lactic acid bacteria (NSLAB) which dominate cheese microbiota during ripening. They tolerate the hostile environment well and strongly influence the biochemistry of curd ripening, contributing to the development of the final characteristics of cheese (Settanni and Moschetti, 2010). In the batches 2 and 4, which were the batches that included in the co-culture the *Lb. plantarum* TAUL 1588 strain, the highest counts were observed at 90 days while in the batch 3 manufactured with the co-culture that included the *Lb.* casei subsp. *casei* SS1644 strain the highest counts of lactobacilli were detected after 2 days and then decreased slightly until the end of the ripening time. In batch 1, lactobacilli were not detected until 180 days of ripening and with values much lower than the other batches since it was the only batch in which no strains of *Lactobacillus* were included in the co-culture used.

The *Enterobacteriaceae* counts are an indicator of the hygienic conditions applied during the cheese-making. The good sanitary quality of the four batches was evidenced because *Enterobacteriaceae* counts were detected at 2 days of ripening with values lower than 4 log CFU/mL. It was observed a significant negative correlation between *Enterobacteriaceae* counts and acidity ($\rho = -0.73$; $P \leq 0.01$) as well as with the salt/moisture ratio ($\rho = -0.95$; $P \leq 0.01$) coinciding with that described by other authors in sheep cheese (Piras et al., 2013).

Table 2
Changes in microbial counts (log CFU/mL) of four sheep milk cheese batches throughout ripening.

Microbial group	Batch[a]	Milk[b]	Ripening time (days)				*P*-value[c]		
			2	90	180	240	R	B	R*B
Aerobic mesophilic microbiota	1	6.80 ± 0.96[a]	9.55 ± 0.04[a]	7.31 ± 0.04[a]	7.17 ± 0.11[a]	7.17 ± 0.12[a]	***	***	***
	2	7.18 ± 0.11[a]	9.68 ± 0.53[b]	8.74 ± 0.28[b]	7.97 ± 0.14[b]	7.84 ± 0.42[b]	***	***	***
	3	7.13 ± 0.07[a]	9.19 ± 0.10[c]	10.92 ± 0.25[c]	9.19 ± 0.11[c]	8.98 ± 0.11[c]	***	***	***
	4	7.36 ± 0.22[a]	9.59 ± 0.27[a]	8.95 ± 0.35[d]	8.47 ± 0.04[d]	8.33 ± 0.04[d]	***	***	***
Lactic acid bacteria	1	6.87 ± 0.52[a]	7.50 ± 0.18[a]	7.36 ± 0.18[a]	7.03 ± 0.18[a]	6.71 ± 0.18[a]	***	***	**
	2	7.14 ± 0.12[a]	9.77 ± 0.41[b]	8.73 ± 0.18[b]	7.74 ± 0.07[b]	7.65 ± 0.27[b]	***	***	**
	3	7.10 ± 0.05[a]	9.20 ± 0.05[c]	8.87 ± 0.37[b]	8.89 ± 0.07[c]	8.75 ± 0.07[c]	***	***	**
	4	7.38 ± 0.10[a]	9.70 ± 0.11[b]	8.83 ± 0.18[b]	8.36 ± 0.28[d]	8.24 ± 0.20[d]	***	***	**
Lactobacilli	1	–	–	–	3.40 ± 0.20[a]	2.40 ± 0.10[a]	***	***	***
	2	5.99 ± 0.04[a]	7.93 ± 0.81[a]	8.71 ± 0.07[a]	7.95 ± 0.78[b]	7.54 ± 0.35[b]	***	***	***
	3	5.40 ± 0.04[a]	9.28 ± 0.17[b]	7.60 ± 0.36[b]	7.22 ± 0.13[c]	7.03 ± 0.20[c]	***	***	***
	4	7.30 ± 0.07[b]	7.77 ± 0.42[c]	8.70 ± 0.25[a]	7.80 ± 0.22[d]	6.83 ± 0.15[d]	***	***	***
Enterobacteriaceae	1	–	3.52 ± 0.22[a]	–	–	–	***	***	***
	2	2.58 ± 0.50	3.57 ± 0.27[a]	–	–	–	***	***	***
	3	–	3.98 ± 0.71[a]	–	–	–	***	***	***
	4	–	2.77 ± 0.35[b]	–	–	–	***	***	***

Results expressed as mean values ± standard deviation, n = 2.
[a-d] Different superscript letters in the same column denote significant statistical differences ($P < 0.05$) between cheese batches.
[a] 1: Lactococcus lactis subsp. lactis TAUL 238 + Lactococcus lactis subsp. cremoris TAUL 1239; 2: starter 1 + Lactobacillus plantarum TAUL 1588; 3: starter 1 + Lactobacillus casei subp. casei SS 1644; 4: starter 1 + Lactobacillus plantarum TAUL 1588 + Lactobacillus casei subp. casei SS 1644.
[b] Pasteurized milk + each culture (0 h).
[c] R: ripening time fixed effect; B: batch fixed effect; R*B: interaction between the fixed effects. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

3.3. Free amino acid content and microstructure of sheep milk cheeses during ripening

The evolution of total free amino acids (TFAAs) during ripening in the four sheep milk cheeses manufactured with different co-cultures is shown in Table 3. The TFAAs concentrations in all the batches increased significantly ($P \leq 0.001$) throughout the ripening period, from 5031.25 mg/kg cheese at 2 days up to 55863.41 mg/kg cheese at 240 days of ripening. This increase in the TFAAs concentrations was mainly due to the proteolytic activity of the strains which made up the co-cultures (González et al., 2015; Herreros et al., 2003). The large proteolytic activity shown by the strains gave rise that the TFAA values observed in the present study were higher than those reported by Poveda et al. (2004) for sheep milk cheese manufactured with different starters and analyzed during 150 days of ripening.

The proteolysis that took place during the cheese ripening can be visually evidenced by the images (Fig. 2) obtained when the microstructure of the different batches was analyzed using confocal laser scanning microscopy. The images of the four cheese batches were very similar. For this reason, in Fig. 2 only the microstructure of batch 1 is shown at 2, 90, 180 and 240 days of ripening. At 2 days of ripening, a continuous and fibrous protein matrix containing irregularly shaped fat globules was observed (Fig. 2 A and E). The fat and protein phases had a slight linear orientation as was also observed by Auty et al. (2001) in Cheedar cheese, attributing this fact to the pressing stage. As the ripening time progressed, changes in the microstructure of cheese could be observed. On the one hand, the protein matrix gradually presented an amorphous structure that could be due to the proteolysis produced by LAB during ripening (Fig. 2A–D). On the other hand, the coalescence of fat globules generated large fat particles and the lipolytic activity during ripening could contribute to the output of free fat (Fig. 2 H). These results were consistent with those reported by other authors for the microstructure of cheese (Everett and Auty, 2008; O'Reilly et al., 2003). It was also shown the physical holes that were generated during ripening (Fig. 2 C, D, G and H).

The different co-cultures used for cheese-making in the present study had a significant ($P \leq 0.001$) effect on the TFAAs concentrations (Table 3). Batch 3 manufactured with the *Lb. casei* subsp. *casei* SS1644 strain showed the highest TFAAs concentrations throughout the ripening period. After 90 days of ripening, this batch showed a TFAAs concentration similar to that shown by the other batches at the end of ripening. Azarnia et al. (2006) have indicated that ripening is a relatively expensive process for the cheese industry. Therefore, the use of this co-culture could reduce the ripening time of sheep milk cheese,

Table 3
Total free amino acid concentration (mg/kg cheese) in sheep milk cheeses manufactured with different co-cultures throughout ripening.

Ripening time (d)	Batch[a]				*P*-value[b]		
	1	2	3	4	B	R	B*R
2	4924.12 ± 53.59[a]	4530.12 ± 49.23[b]	5910.09 ± 53.17[c]	4760.68 ± 46.64[a]	***	***	***
90	16877.92 ± 170.46[a]	16687.85 ± 158.53[a]	40320.11 ± 450.44[b]	16978.97 ± 187.61[a]	***	***	***
180	34563.46 ± 307.75[a]	34763.36 ± 360.19[a]	79662.49 ± 771.78[b]	38414.89 ± 414.78[a]	***	***	***
s	44671.13 ± 478.42[a]	42076.23 ± 451.02[a]	87623.26 ± 928.00[b]	49083.01 ± 473.76[c]	***	***	***

Results as mean values ± standard deviation, n = 4.
[a-d] Different lowercase superscript letters in a same row denote significant statistical differences ($P < 0.05$) between cheese batches.
[a] 1: Lactococcus lactis subsp. lactis TAUL 238 + Lactococcus lactis subsp. cremoris TAUL 1239; 2: starter 1 + Lactobacillus plantarum TAUL 1588; 3: starter 1 + Lactobacillus casei subp. casei SS 1644; 4: starter 1 + Lactobacillus plantarum TAUL 1588 + Lactobacillus casei subp. casei SS 1644.
[b] B: batch fixed effect; R: ripening time fixed effect; B*R: interaction between the fixed effects. $^{***}P \leq 0.001$.

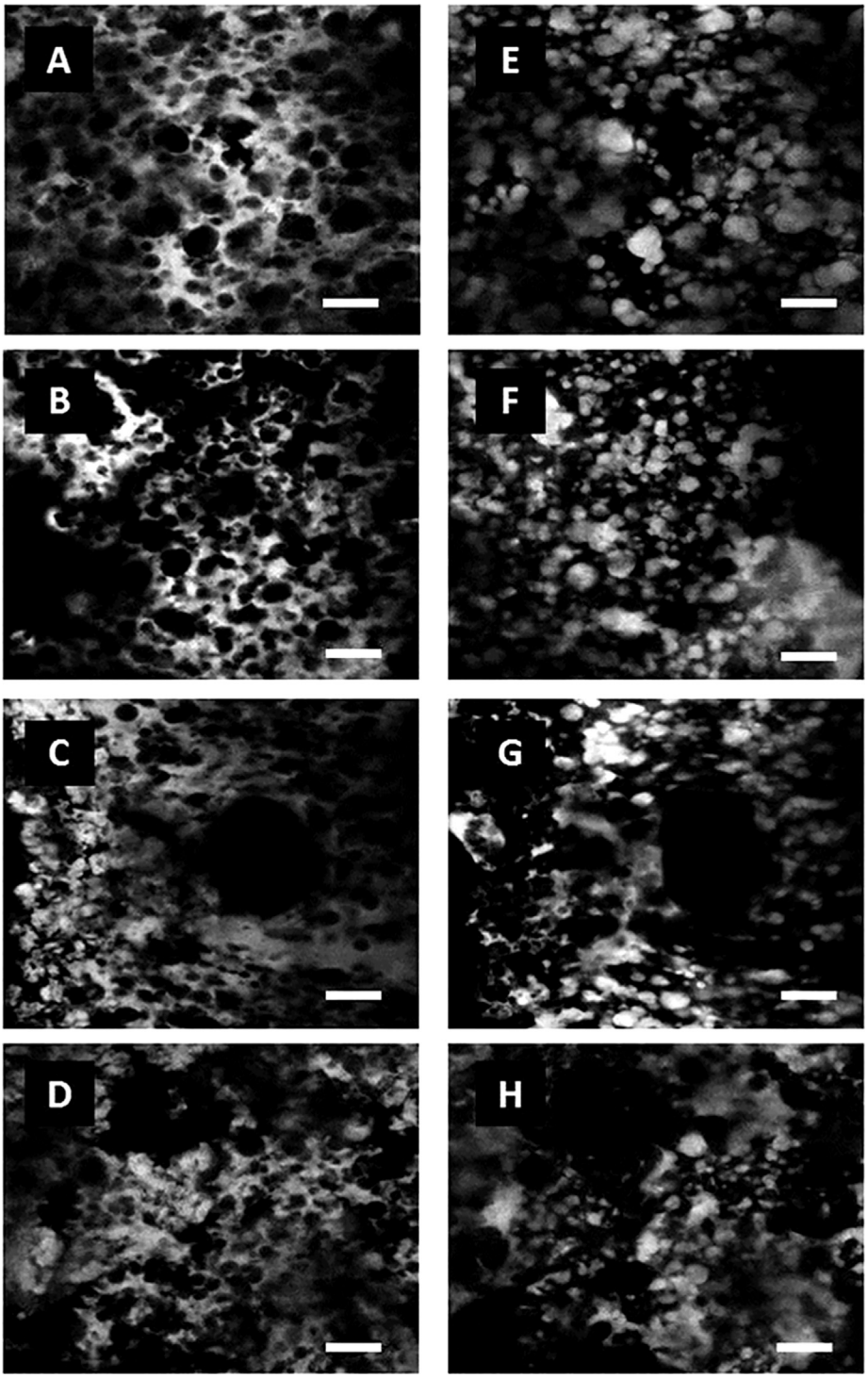

Fig. 2. Confocal laser scanning images of the protein (A–D) and fat (E–H) in cheese batch 1 throughout the ripening period: 2 days (A and E), 90 days (B and F), 180 days (C and G) and 240 days (D and H). The protein and fat are shown as light areas against a dark background. Scale bar, 25 μm.

providing technological benefits.

Batches 1, 2 and 4 did not show significant differences ($P \geq 0.05$) in the TFAAs concentration until the end of ripening. At 240 days, batch 2 made with *Lc. lactis* strains plus *Lb. plantarum* TAUL1588 presented a similar concentration of TFAAs to that of batch 1 made with the *Lc. lactis* strains. However, batch 4 manufactured with the four autochthonous strains had higher TFAAs concentration than batches 1 and 2 but lower than batch 3.

The individual concentration of 21 free amino acids in sheep milk cheeses made with different co-cultures throughout the ripening period is shown in Fig. 3. The concentration of most of the free amino acids studied increased significantly ($P \leq 0.001$) during the cheese ripening. In general, the most abundant free amino acids were leucine, glutamic acid, phenylalanine, proline, alanine and valine which accounted approximately the 60% of the TFAAs. Tyrosine, histidine, glycine, tryptophan and arginine were minor free amino acids representing less than the 5% of the TFAAs. The data available on the literature about the major free amino acids and their concentration in sheep milk cheese varies widely (Madrau et al., 2006; Mangia et al., 2008; Poveda et al., 2015). This fact could be mainly due to the different lactic acid bacteria

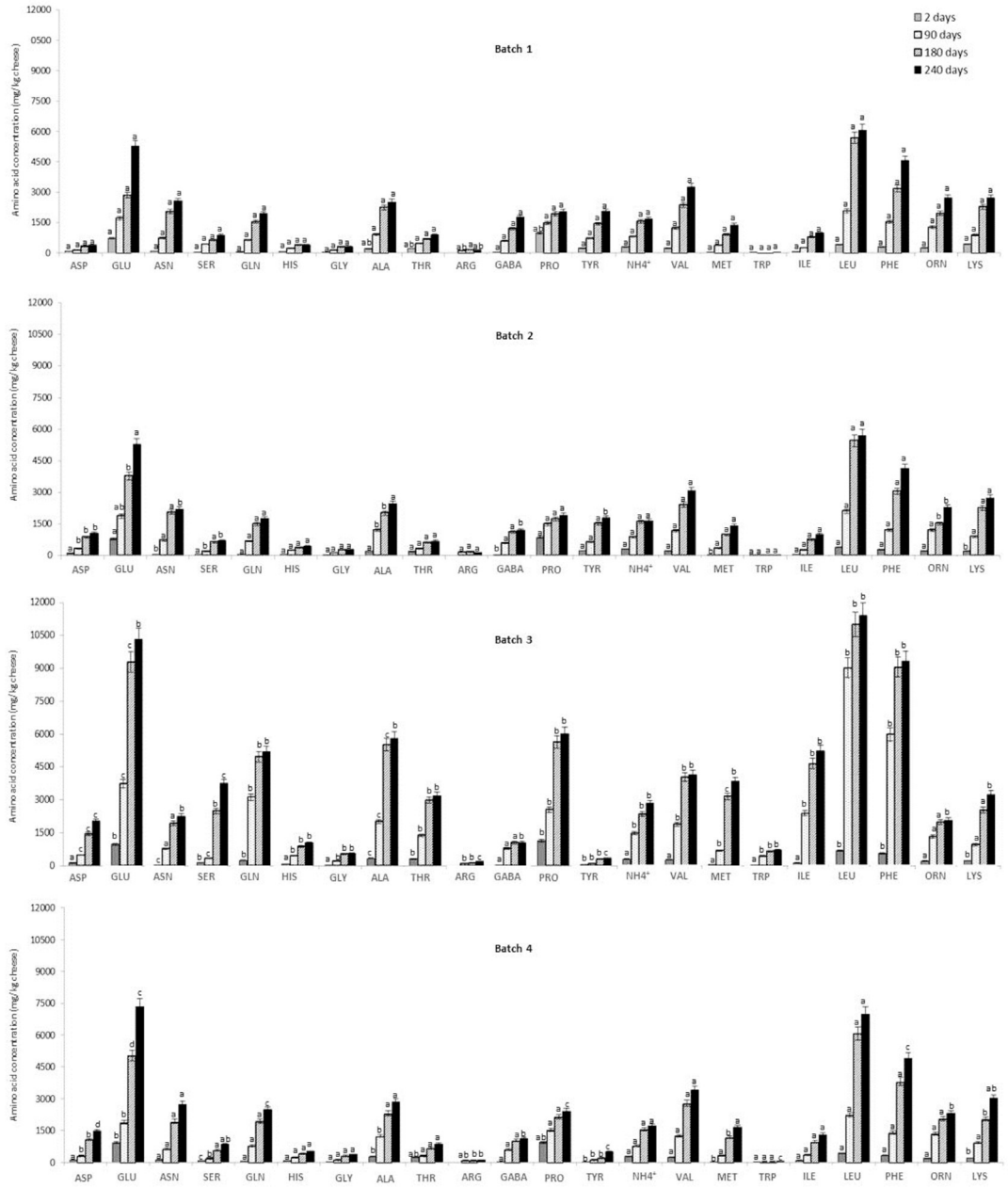

Fig. 3. Evolution of the individual free amino acids during ripening of sheep milk cheese manufactured with different co-cultures: (Batch1) cheese made with starter composed of *Lactococcus lactis* subsp. *lactis* TAUL 238 and *Lc. lactis* subsp. *cremoris* TAUL 1239 strains; (Batch 2) cheese made with starter 1 and *Lactobacillus plantarum* TAUL 1588; (Batch 3) cheese made with starter 1 and *Lactobacillus casei* subsp. *casei* SS 1644; (Batch 4) cheese made with starter 1 and both *Lactobacillus* strains used in batches 3 and 4.
Data represent mean ± standard deviation (indicated by vertical error bars), n = 4. Different letters (a-d) in the same bar of the diagram for each free amino acid in the same ripening time indicate significant statistical differences ($P < 0.05$) between cheese batches.
ASP: aspartic acid; GLU: glutamic acid; ASN: asparagine; SER: serine; GLN: glutamine; HIS: histidine; GLY: glycine; ALA: alanine; THR: threonine; ARG: arginine; GABA: gamma-aminobutyric acid; PRO: proline; TYR: tyrosine; VAL: valine; METH: methionine; TRYP: tryptophan; ISOLEU: isoleucine; LEU: leucine; PHENYL: phenylalanine. ORNI: ornithine; LYS: lysine.

(LAB) used for cheese-making since LAB present a complex proteolytic system formed by different proteinases and peptidases depending on the LAB specie (Fox et al., 2016). As Poveda et al. (2004) have indicated the composition of the caseins is different; α_{S1}-casein has a high content of leucine, phenylalanine and valine, while β-casein has high content of proline. In addition free amino acids are released by the action of peptidases that vary among different strains. Therefore, depending on the specific substrates of the enzymes present in the proteolytic system of LAB, the type and concentration of free amino acids in cheese will be different.

The results obtained in the present study are relevant since little information is available about the effect of different autochthonous co-cultures on the concentration of GABA and ornithine in sheep milk cheese during ripening. GABA and ornithine have been reported as non-protein amino acids with numerous physiological functions (Diana et al., 2014b). GABA could be synthesized from L-glutamate by the glutamate decarboxylase (GAD) present in some LAB (Cotter and Hill, 2003; Lacroix et al., 2013). Milk caseins do not present GABA but they have a high content in L-glutamate (17.5% of the TFAAs) which is released during cheese ripening and can be metabolized to GABA by the

action of LAB (Hejtmánková et al., 2012). In Fig. 3 it is shown that GABA concentration in the four cheese batches increased more than eighty-fold from 2 days to 240 days of ripening. The major concentration of GABA was observed at 240 days of ripening in Batch 1 followed by the other three cheese batches which did no present differences ($P \geq 0.05$) between them. The importance of this study falls on the high concentration of GABA found in all the cheese batches at the end of the ripening time. Regarding the dose of GABA which is in the health-promoting range, it has been observed that a daily intake of 50 g of experimental cheese (containing 16 mg of GABA) decreased blood pressure in humans (Pouliot-Mathieu et al., 2013). In the present study 50 g of batch 1 and 50 g of the other three batches would suppose 90 mg and 60 mg of GABA, respectively. This fact implied that small portions of these cheeses would be necessary to achieve the physiological effect indicated above.

Presence of ornithine in foods is gaining attention since some studies have shown its bioactive functions on human health (Kurata et al., 2012; Sugino et al., 2008). Currently, there is no information about the effective dose of ornithine to achieve the reported physiological benefits, what is known is that ornithine can be synthetized by the enzymatic activity of LAB metabolism through the precursors arginine and citrulline during cheese ripening (Diana et al., 2014b). In the four sheep milk cheeses there was an increase ($P \leq 0.001$) in the values of ornithine throughout the ripening time. No significant ($P \geq 0.05$) differences were observed between the four batches analyzed until 240 days of ripening. As in the case of GABA, batch 1 was the one with the highest concentration of ornithine. Batches 2, 3 and 4 did no show differences ($P \geq 0.05$) between them. These concentrations were higher than those (ranged between 0.40 and 1.08 g/kg) described by Diana et al. (2014b) for sheep milk cheeses. These results open the possibility of carrying out further studies with these co-cultures for the possible development of functional cheeses.

3.4. Biogenic amines content of sheep milk cheeses during ripening

During cheese ripening, free amino acids were generated by the autochthonous co-cultures studied. Some of these free amino acids can act as substrate for secondary catabolic reactions by the decarboxylase activity of LAB. The result of these metabolic routes can be other free amino acids such as GABA and ornithine which have beneficial effects on human health or biogenic amines which consumption in elevated concentrations could have negative effects on human health when the organism in not able to degrade them though the action of monoamine and diamine oxidases (Broadley, 2010; EFSA, 2011; Manca et al., 2015). For this reason, the effect of the different co-cultures used and the ripening time on the biogenic amines content of the cheese batches was also analyzed.

The total biogenic amines content of the sheep milk cheeses manufactured with different co-cultures increased significantly ($P \leq 0.001$) during ripening, ranging between 10.17 mg/kg cheese for batch 1 at 2 days and 820.18 for batch 3 at 240 days (Table 4). These values were similar to those described for sheep milk cheeses (Renes et al., 2014; Schirone et al., 2013). The maximum permitted concentration of biogenic amines in dairy products has not been established yet from a legislative point of view. Most studies have concentrated on the study of histamine and tyramine, as these are the biogenic amines most often associated with food poisoning and the most abundant in sheep milk cheese (Linares et al., 2016). In the present study, histamine was not detected in all the cheese batches (the detection limit for histamine was 1.78 mg/kg cheese). Tyramine concentrations ranged between 308.65 mg/kg cheese and 585.47 mg/kg cheese at the end of ripening, being within the maximum tolerable limits (100 mg/kg – 800 mg/kg) reported by ten Brink et al. (1990). Nonetheless, it is necessary to highlight that cadaverine and putrescine which have not been associated with food poisoning, may enhance the toxicity of histamine and tyramine. In this sense, the total biogenic amines content should not exceed the amount of 900 mg/kg established by Valsamaki et al. (2000). In this regard, none of the samples exceeded the indicated limit, not representing a risk to the consumer's health.

In Table 4, it can be observed that batch 3, manufactured with the co-culture containing the *Lb. casei* subsp. *casei* SS1644 strain, was the batch that presented the highest concentration of total biogenic amines throughout the ripening time. It was also the batch that had the highest concentration of TFAAs (Table 3). These results are consistent with those reported by Novella-Rodriguez et al. (2003) who indicated that an increase in cheese proteolysis during ripening could produce an increase in biogenic amines content. Batch 2 made with the *Lb. plantarum* TAUL1588 strain presented the lowest concentration of total biogenic amines, followed by batches 1 and 4.

The information available about the major biogenic amines in sheep cheese is variable, as in the above-case of the TFAAs, because the type and concentration of biogenic amines found in cheese depend on the cheese variety and on the multiple factors involved in the formation and accumulation of these biogenic amines (Renes et al., 2014). The individual biogenic amines concentration in sheep milk cheese batches manufactured with different co-cultures during ripening are shown in Table 4. Histamine and tryptamine were not detected in the batches examined. Tyramine resulted to be the biogenic amine in the highest concentration throughout the ripening time. At the beginning of ripening, it was not detected in batches 1 and 2. Batch 3 was the one that showed the highest concentration of tyramine throughout the ripening time, followed by batch 4.

Putrescine and phenylethylamine were no detected in batches 1 and 2 during ripening and in batch 4 at the beginning of ripening. Batch 3 presented the highest concentration of these amines at 2, 90, 180 and 240 days of ripening. It was observed an increase in the concentration of these amines of 9 fold for putrescine and 6 fold for phenylethylamine at 240 days of ripening with respect 2 days. As it has been described previously, some biogenic amines may cause undesirable toxicological effects. However, in the case of the phenylethylamine, it has been observed that it has beneficial physiological effects on human health (Irsfeld et al., 2013).

Batches 1 and 2 had the lowest cadaverine concentration without detecting differences ($P \geq 0.05$) between them. The concentration of cadaverine in these batches was not affected by the ripening time ($P \geq 0.05$). This same trend was observed in batch 4 until 180 days of ripening at which point the cadaverine concentration increased. Batch 3 showed the highest concentration of cadaverine during cheese ripening. The concentration of this amine increased significantly ($P \leq 0.001$) from 2 days of ripening to 90 days, moment from which it increased slightly until the end of ripening.

4. Conclusions

Consumer's demand for healthy foods is leading the dairy industry to develop foods in which the nutritional quality has been improved. The co-culture formed by autochthonous *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* as starters and *Lb. plantarum* as adjunct culture could be a good approach to the development of functional sheep milk cheeses with reduced biogenic amine content.

As cheese ripening is a technological stage that involves the investment of a lot of money, the co-culture containing the four autochthonous LAB strains of the present study could be an alternative to reduce the ripening time of sheep milk cheeses and it also could produce high concentrations of bioactive compounds such as GABA and ornithine in cheese.

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Table 4
Concentration (mg/kg cheese) of biogenic amines in sheep milk cheeses manufactured with different co-cultures throughout ripening.

Biogenic amine	Ripening time (d)	Batch[a]				*P*-value[b]		
		1	2	3	4	B	R	B*R
Tyramine	2	nd	nd	71.47 ± 0.17^{a}	44.04 ± 0.14^{b}	***	***	***
	90	342.95 ± 2.13^{a}	274.29 ± 1.81^{b}	408.60 ± 2.05^{c}	415.58 ± 4.05^{c}	***	***	***
	180	356.67 ± 3.21^{a}	288.08 ± 2.88^{b}	451.27 ± 2.02^{c}	436.09 ± 8.69^{d}	***	***	***
	240	493.85 ± 2.47^{a}	308.65 ± 1.54^{b}	585.47 ± 11.47^{c}	539.29 ± 11.69^{d}	***	***	***
Putrescine	2	nd	nd	8.27 ± 0.01	nd	***	**	***
	90	nd	nd	72.56 ± 0.09	nd	***	**	***
	180	nd	nd	72.56 ± 0.09	nd	***	**	***
	240	nd	nd	75.45 ± 0.11^{a}	56.76 ± 0.10^{b}	***	**	***
Cadaverine	2	10.17 ± 0.01^{a}	10.20 ± 0.01^{a}	56.20 ± 0.06^{b}	10.20 ± 0.01^{a}	***	**	***
	90	10.19 ± 0.04^{a}	10.20 ± 0.03^{a}	84.29 ± 0.20^{b}	10.21 ± 0.01^{a}	***	**	***
	180	10.33 ± 0.02^{a}	10.20 ± 0.01^{a}	84.47 ± 0.20^{b}	10.22 ± 0.01^{a}	***	**	***
	240	10.40 ± 0.02^{a}	10.21 ± 0.01^{a}	89.47 ± 0.21^{b}	31.65 ± 0.31^{c}	***	**	***
Phenylethylamine	2	nd	nd	11.99 ± 0.01	nd	***	**	***
	90	nd	nd	60.54 ± 0.26^{a}	24.23 ± 0.02^{b}	***	**	***
	180	nd	nd	65.31 ± 0.55^{a}	30.54 ± 0.02^{b}	***	**	***
	240	nd	nd	69.79 ± 0.75^{a}	45.29 ± 0.06^{b}	***	**	***
Total	2	10.17 ± 0.01^{a}	10.20 ± 0.02^{a}	147.93 ± 0.25^{b}	54.24 ± 0.15^{c}	***	***	***
	90	353.12 ± 2.34^{a}	284.49 ± 1.28^{b}	625.99 ± 1.27^{c}	450.02 ± 1.00^{d}	***	***	***
	180	367.00 ± 1.58^{a}	298.28 ± 0.47^{b}	673.61 ± 1.69^{c}	476.85 ± 1.67^{d}	***	***	***
	240	504.25 ± 0.71^{a}	318.86 ± 0.47^{b}	820.18 ± 4.00^{c}	672.99 ± 2.94^{d}	***	***	***

Results expressed as mean values ± standard deviation, n = 4.
[a-d] Different lowercase superscript letters in a same row denote significant statistical differences ($P < 0.05$) between cheese batches cheese, respectively.
[2]B: batch fixed effect; R: ripening time fixed effect; B*R: interaction between the fixed effects. **$P \leq 0.01$; ***$P \leq 0.001$.
nd: not detected; limit of detection for tyramine, putrescine and phennylethylamine was 0.86, 0.41, 0.26 and 1.53 mg kg^{-1}
[a] 1: Lactococcus lactis subsp. lactis TAUL 238 + Lactococcus lactis subsp. cremoris TAUL 1239; 2: starter 1 + Lactobacillus plantarum TAUL 1588; 3: starter 1 + Lactobacillus casei subp. casei SS 1644; 4: starter 1 + Lactobacillus plantarum TAUL 1588 + Lactobacillus casei subp. casei SS 1644.
[b] B:batch effect; R: ripening effect; B*R: interaction batch and ripening time effect.

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